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PRINCIPAL INVESTIGATOR: Erin O'Neill, B.S.

CONTRACTING ORGANIZATION: University of Chicago
Chicago, IL 60637

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Erin O'Neill, B.S.

E-Mail: eionson@uchicago.edu

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14. ABSTRACT: The ER modulates various signaling cascades involved in the survival and differentiation of a wide range of tissues, both reproductive and nonreproductive. In our studies, we confirmed that 17ß-estradiol (E2) and other ER-specific ligands rapidly phosphorylate ERK1/2 in breast cancer and neuronal cell lines. Subsequently, we found that E2 rapidly stimulates aCaMKII autophosphorylation in immortalized GnRH neurons and primary hippocampal neurons in a calmodulin and Ca2+ influx-dependent manner. Interestingly, ERa associates with aCaMKII and their interaction attenuates the positive effect of E2 on aCaMKII autophosphorylation, suggesting that ERa plays a complex role in modulating aCaMKII activity. However, it appears that the activating signal of E2 is dominant since there is a clear, positive downstream response to E2-activated aCaMKII; pharmacological inhibitors and RNAi technology demonstrated ERa-mediated aCaMKII signaling targets ERK1/2, CREB, and MAP2 for phosphorylation. In vivo, E2 or PPT administration to ovariectomized female rats significantly enhances aCaMKII activity in the hippocampus after 1 hr or 24 hr of exposure. Additionally, E2 administration induces ERK1/2 phosphorylation in vivo in the uterine horn and brain extracts. Functionally, E2-induced aCaMKII signaling influences neurite outgrowth of primary hippocampal neurons. Our findings suggest a novel model for the modulation of aCaMKII signaling by ERa, which provides a molecular link as to how E2 might influence brain function. Ultimately, the characterization of this signaling pathway could be exploited to create new selective estrogen receptor modulators that enhance cognitive function in postmenopausal women without affecting breast tissue or increasing the risk of developing breast cancer.

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Introduction:

Estrogen receptor (ER)-mediated extranuclear signaling is involved in the survival, growth, and differentiation of ER-expressing cells and tissues, both reproductive and nonreproductive (Manolagas, 2001; Levin, 2005). We have pursued this research in order to gain a better understanding of rapid estrogen action on targeted cytoplasmic signaling cascades, including the mitogen-activated protein kinase (MAPK) and Ca^{2+}/calmodulin-dependent kinase II (CaMKII) pathways. CaMKII is a multi-subunit kinase that is exquisitely responsive to Ca^{2+} levels and consists of 4 distinct isoforms, α-, β-, δ-, and γCaMKII, each of which is comprised of the same major protein domains, including the catalytic, autoinhibitory, and self-association domains. δ- and γCaMKII have a relatively ubiquitous expression pattern while α- and βCaMKII are prevalent in brain tissue; in fact, αCaMKII comprises approximately 2% of total protein in the rat forebrain (Schulman, 1992). αCaMKII, in particular, has been shown to be essential for the induction of long-term potentiation (LTP) (Manilow, 1989; Silva, 1992; Wang, 2006), which is thought to be the molecular mechanism underlying learning and memory. It is also involved in neurotransmitter regulation (Stefani G, 1997; Liu R, 2005), as well as neuronal survival and differentiation (Blanquet P, 1999). Therefore, it is important to understand the relationship between rapid E2 action and αCaMKII signaling in order to gain insight into how estrogen affects cognitive function. Our studies better define the mechanism responsible for ER-mediated αCaMKII and ERK1/2 signaling, and examine it in an animal model. Specifically, the observed E2-induced αCaMKII/ERK1/2 phosphorylation was characterized in breast cancer cells, immortalized gonadotropin releasing hormone (GnRH) neurons, cultured embryonic primary hippocampal neurons, and ovariectomized immature female rats. We also explored the downstream signaling and cellular events resulting from E2-stimulated αCaMKII/ERK1/2 signaling. Together, these findings demonstrate that ERα plays a complex role in the regulation of αCaMKII activity. A more comprehensive understanding of this signaling is required for the development of selective estrogen receptor modulators (SERMs) that possess beneficial agonist activity in the brain and antagonist activity in breast tissue to reduce the risk of developing breast cancer in postmenopausal women.

Body:

Our preliminary evidence included confirmation that 17β-estradiol (E2), diethylstilbestrol (DES), propyl pyrazole triol (PPT), and 4-estren-3α-17β-diol (Estren) rapidly phosphorylate and activate ERK1/2 in the breast cancer cell line, MCF-7, and that this effect is effectively blocked by the potent ER antagonist, ICI 182, 780 (ICI). We then used immature ovariectomized female rats as an animal model and showed that E2 can elicit ERK1/2 phosphorylation *in vivo* in both the uterine horn and brain after intraperitoneal (I.P.) injection (Figs. 1 and 2) and that E2 administration can also induce αCaMKII autophosphorylation in the brain (Fig. 3). We identified αCaMKII as an upstream regulator of rapid E2-induced ERK1/2 phosphorylation in immortalized NLT GnRH neurons (Fig. 8A), which gave us our first glimpse of a novel mechanism by which ER can signal to ERK1/2.

We continued our investigation of ER-mediated ERK1/2 phosphorylation with a closer examination of CaMKII signaling. CaMKII is a ubiquitous kinase that a former

graduate student in the lab previously demonstrated to interact with ERα in breast cancer cells. She also showed that CaMKII can phosphorylate ER in the ligand-binding domain, a modification that enhances the receptor's ability to function as a transcription factor in breast cancer cells. As we continued to obtain inconsistent data with MCF-7 cells, we focused on the NLT GnRH neurons as a cellular model. To examine the effect of E2 on αCaMKII activation it was first established that ERα is expressed in NLT immortalized GnRH neurons via immunofluorescence (Fig. 4A) and western blot analysis (Fig. 4B). Additionally, a luciferase reporter assay in which an estrogen response element (ERE) is linked to luciferase (3ERE-Luc) shows that NLT cells transfected with the 3ERE-Luc reporter plasmid have significantly increased luciferase activity when treated with either E2, propyl pyrazole triol (PPT: ERα-selective agonist), or diarylpropionitrile (DPN: ERβ-selective agonist) but not with vehicle or in the presence of ICI 182, 780 (ICI: potent ERα/β antagonist), confirming that NLT cells express functional ERα and ERβ (Fig. 4C). The autophosphorylation of αCaMKII was also examined over time, and the peak of E2-induced kinase autophosphorylation occurs at 10 min of treatment. As a positive control a combination of forskolin and A23187 (F/A) was used to ultimately increase intracellular Ca^{2+} levels (Fig. 4D). This effect is blocked by pre-treatment with KN-62, a CaMKII-specific inhibitor, or ICI as shown by Western blot analysis (Fig. 4E).

Immunofluorescence studies demonstrated that in NLT cells, E2 stimulates αCaMKII activation in the cytoplasm and cell outgrowths within 10 min compared to unstimulated cells, and KN-62 pre-treatment counteracts the E2 effect (Fig. 5A). E2 induces kinase autophosphorylation in 43.10±8.49% of cells, whereas only 8.31±2.88% of unstimulated cells and 20.54±12.81% of E2+KN-62-treated cells have autophosphorylated αCaMKII in the cytoplasm. In contrast, the nuclear pool of αCaMKII in NLT cells appears to be activated regardless of the treatment. Fractionation of NLT cells after 10 min of ligand treatment confirmed that E2-induced αCaMKII autophosphorylation occurs primarily in the cytosolic fraction, which is considerably decreased by KN-62 (Fig. 5B).

To understand the mechanism by which E2 influences αCaMKII autophosphorylation we examined the hypothesis that Ca^{2+} signaling is involved since E2 rapidly triggers Ca^{2+} influx and mobilization in neuronal cells (Wu, 2005; Zhao, 2005). NLT cells were either unstimulated or treated for 10 min with vehicle, E2 alone, or E2 with a 30 min pre-treatment of BAPTA-AM (intracellular Ca^{2+} chelator), W7 (CaM inhibitor), or Nifedipine (L-type voltage-gated Ca^{2+} channel antagonist) (Fig. 5C/D). Western blot analysis demonstrated that E2-induced αCaMKII autophosphorylation is dependent on both intracellular Ca^{2+} and CaM action as it is completely blocked by BAPTA-AM and W7 pre-treatment. Ca^{2+} influx through L-type Ca^{2+} channels is also clearly involved as Nifedipine significantly decreases αCaMKII autophosphorylation induced by E2. Treatment with BAPTA-AM, W7, and Nifedipine alone had no effect on αCaMKII autophosphorylation (data not shown). These data suggest that E2-induced αCaMKII autophosphorylation is the result of Ca^{2+} influx via L-type Ca^{2+} channels, which can then complex with CaM to stimulate αCaMKII activity.

As both ERα and ERβ are expressed in a variety of brain regions (Shughrue, 1997) and in NLT cells (Fig. 4C), it is important to understand if one or both of them could be involved in stimulating αCaMKII autophosphorylation. To address this question, we first examined the effect of two ER-selective ligands, PPT, an ERα-selective

agonist, and DPN, an ERβ-selective agonist. Western blot analysis demonstrated that while PPT is able to rapidly induce αCaMKII autophosphorylation in a dose-dependent manner, DPN is unable to elicit the same response even at the highest dose (Fig. 6A). Additionally, we found that E2 treatment is unable to induce αCaMKII autophosphorylation at any of the time points examined in SK-N-SH cells, which is a neuroblastoma cell line that we have found to be void of ERα but contains functional ERβ as evidenced by reporter assays (Fig. 6B). Finally, we investigated the effect of rapid E2 action on Cos7 cells that were transfected with αCaMKII alone or in the presence of either FLAG-ERα or FLAG-ERβ. The co-expression of ERα but not ERβ with αCaMKII results in increased kinase autophosphorylation after just 2 min of E2 but not vehicle treatment. Importantly, αCaMKII expression alone is inadequate for E2-induced kinase activation while it retains the ability to autophosphorylate in response to calcium mobilization stimulated by forskolin and A23187 treatment (Fig. 6C). These data suggest that ERα is the receptor subtype responsible for αCaMKII autophosphorylation induced by E2 treatment of immortalized neuronal and co-transfected Cos7 cells.

Examination of the αCaMKII amino acid sequence revealed that it contains a nuclear receptor interaction motif, or NR box, within the CaM-binding region of the autoinhibitory domain. If αCaMKII can interact with ERα via this NR box, an LTTML sequence, mutation of the consensus sequence should impair the association. GST pulldown studies show that when the NR box of αCaMKII is disrupted by the mutation L304A, the E2-dependent interaction observed between wild type αCaMKII and GST-ERα-LBD (ligand binding domain) is completely abolished (Fig. 7A). To understand if the kinase binds to ER in a manner similar to that of a typical nuclear receptor coactivator, the ability of αCaMKII to bind various GST-ERα-LBD mutants was examined. It was previously reported that an array of point mutations in the ERα-LBD (ERα-I358R, V376R, and E542K) disrupt the binding of the glucocorticoid receptor interacting protein (GRIP) and steroid receptor coactivator 1 (SRC-1) to the hydrophobic binding pocket of ERα (Feng, 1998). Interestingly, αCaMKII is unable to bind any of the GST-ERα-LBD mutants even in the presence of E2, suggesting that αCaMKII binds to the same hydrophobic pocket as other typical coactivator proteins (Fig. 7B). Additionally, no association is observed in the presence of tamoxifen (T) in figure 7A because this ligand typically induces the antagonist conformation of ERα-LBD, which would inhibit the binding of αCaMKII to the hydrophobic pocket of the receptor.

To test if the two proteins interact in cells, Cos7 cells were co-transfected with FLAG-ERα and αCaMKII, and the proteins were then immunoprecipitated with either anti-FLAG or anti-αCaMKII, respectively. Western blot analysis for ERα and αCaMKII showed that the proteins associate only in cells treated with E2 but not vehicle for 10 min (Fig. 7C). To confirm that the ERα-LBD mutant, V376R, is incapable of interacting with αCaMKII as already demonstrated by GST pulldown experiments, Cos7 cells were co-transfected with αCaMKII and either wild-type ERα or ERα-V376R, and ERα was then immunoprecipitated with the ER-specific antibody, H222.2. Expectedly, the mutation of V376R effectively disrupted the E2-dependent interaction between αCaMKII and ERα in cells (Fig. 7D).

Although data in Figure 5C provides evidence that E2-induced αCaMKII autophosphorylation is dependent on Ca^{2+} signaling, it is fair to hypothesize that

αCaMKII can also be directly activated by ERα through its interaction with the autoinhibitory region of the kinase, which could potentially disrupt its autoinhibition much in the same manner as calmodulin. To test this hypothesis, Cos7 cells were co-transfected with αCaMKII and either wild type ERα or the interaction mutant, ERα-V376R. The cells were either unstimulated or treated with E2, or E2+BAPTA-AM pre-treatment, and αCaMKII autophosphorylation was detected by western blot analysis (Fig. 7E/F). Surprisingly, disrupting the interaction of ERα and αCaMKII significantly enhances the ability of E2 to stimulate kinase autophosphorylation (V376R expression versus wild-type expression: ~4-fold increase versus ~2-fold increase in autophosphorylation). Additionally, pre-treatment with BAPTA-AM abolishes E2-induced αCaMKII autophosphorylation when the ERα-αCaMKII interaction is disrupted, implying that E2 can still influence Ca^{2+} signaling to activate αCaMKII even when its interaction with ERα is impaired (Fig. 7G/H). Since the ERα binding site overlaps with the CaM-binding site, these data suggest the possibility that ERα may compete with Ca^{2+}/CaM binding on αCaMKII and decrease its kinase activity, perhaps by locking the kinase subunits in an autoinhibited state. Taken together, these data show that while E2 can stimulate αCaMKII autophosphorylation via Ca^{2+} influx, the association of ERα with αCaMKII negatively regulates this event, perhaps to keep the amount of active kinase in check.

We wanted to confirm that E2 can rapidly stimulate ERK1/2 activation via CaMKII signaling, as well as look at the phosphorylation status of downstream proteins such as CREB, which is a known target for ERK1/2 and αCaMKII action (Wu, 2005). E2 treatment for 10 min to NLT cells results in the phosphorylation of all three proteins in a CaMKII- dependent manner as it is blocked by pre-treatment with KN-62, the specific CaMKII inhibitor. The phosphorylation is also dependent upon Ca^{2+} influx from L-type Ca^{2+} channels as it is inhibited by Nifedipine pretreatment (Fig. 8A/B). Additionally, CREB phosphorylation is dependent on ERK1/2 activation as treatment with the MEK inhibitor U0126 30 min prior to E2 significantly reduces its the phosphorylation level, suggesting that αCaMKII activation is the upstream signaling event. The involvement of CaM was also examined and 30min pre-treatment of NLT cells with calmidazolium, a potent CaM inhibitor, prior to 10min of E2 completely blocks the effect of E2 on ERK1/2 and CREB phosphorylation (Fig. 8C/D), revealing that CaM action is, in fact, important for E2-induced ERK1/2 and CREB activation.

We continued to investigate E2-induced αCaMKII autophosphorylation *in vivo* and administered vehicle (10% cremaphor/2% EtOH in saline), E2 (0.2ug/rat), or PPT (10ug/rat) subcutaneously to ovariectomized female rats for 1hr or 24hr. Immunohistochemistry (IHC) for autophosphorylated αCaMKII and total CaMKII was performed and showed that either E2 or PPT administration enhances αCaMKII autophosphorylation in the hippocampus compared to unstimulated and vehicle-injected animals after 1 hr and 24 hr of treatment (Fig. 9A/B, top panels). After 1hr of exposure, the ERα agonists are able to enhance αCaMKII activity, with the most significant effect in the dentate gyrus (DG). Additionally, PPT increases kinase activity in the CA1, CA2, and CA3 pyramidal neurons while E2 has a minimal impact in these areas (Fig. 9A, middle panels). However, by 24 hr of E2 exposure, αCaMKII autophosphorylation is evident in all of the hippocampal structures examined, with the most striking effect in the CA3 neurons and DG. PPT continues to induce αCaMKII autophosphorylation at 24 hr

in all structures examined (Fig. 9B, middle panels). To verify that the staining is specific, IHC was performed for autophosphorylated αCaMKII in the presence of a specific blocking peptide. The simultaneous use of blocking peptide with antibody completely abolished any staining, indicating that the activity we observed is specific (Fig. 9A/B, bottom panels). Total αCaMKII levels in the hippocampus do not change dramatically with the different treatment conditions (data not shown). These data suggest that E2-mediated αCaMKII autophosphorylation does occur *in vivo* and may be involved in a variety of physiological responses including learning and memory processes.

As αCaMKII is targeted for autophosphorylation by E2 and PPT in the hippocampus *in vivo*, we decided to examine the effect of E2 on CaMKII signaling in cultured embryonic primary hippocampal neurons. E2 treatment for 10 min results in the autophosphorylation of αCaMKII as well as the phosphorylation of ERK1/2, CREB, and microtubule associated protein 2 (MAP2) (Fig. 10). The phosphorylation status of ELK-1 and ERK5 is unaltered by E2 treatment. αCaMKII, ERK1/2, CREB, and MAP2 phosphorylation induced by E2 is effectively blocked by KN-62 which suggests that CaMKII is involved in these signaling events. Inhibition of ER with ICI pre-treatment decreased E2-stimulated αCaMKII autophosphorylation as well as ERK1/2, CREB, and MAP2 phosphorylation, which is in agreement with NLT data. Interestingly, blocking MEK1/2 activity with U0126 pre-treatment decreases αCaMKII autophosphorylation as well as ERK1/2, CREB, and MAP2 phosphorylation by E2, suggesting that MEK1/2 can also function upstream of αCaMKII in these cells, which contradicts what was observed in the NLT GnRH neurons in which E2-stimulated αCaMKII autophosphorylation was unaffected by U0126. Additionally, the effect of Ca^{2+}/CaM signaling was investigated on E2-induced signaling in these neurons. Figure 11 shows that E2-induced ERK1/2, CREB, and MAP2 phosphorylation is severely compromised when Ca^{2+} signaling and CaM action were disrupted with BAPTA-AM or calmidazolium pre-treatment, respectively.

To directly demonstrate the requirement of αCaMKII for the E2-induced phosphorylation of downstream signaling proteins, its protein expression in primary hippocampal neurons was knocked down using specific siRNA oligonucleotides (Fig. 12A). Targeted inhibition of αCaMKII expression was achieved in siαCaMKII-transfected hippocampal neurons as demonstrated by western blot analysis; the total αCaMKII level is knocked down to ~23% of the level in neurons transfected with non-targeting (N.T.) siRNA (Fig. 12B). The siRNA-mediated inhibition of αCaMKII prevents E2-induced αCaMKII autophosphorylation (Fig. 12C) as well as ERK1/2 (Fig. 12D), CREB (Fig. 12E), and MAP2 (Fig. 12F) phosphorylation to the same extent as KN-62 pre-treatment. Together, these results indicate that αCaMKII action mediates the E2-induced phosphorylation of ERK1/2, CREB, and MAP2.

Both αCaMKII and E2 action have been implicated in neurite outgrowth (Williams, 1995; Gollapudi, 2001; Gaudilliere, 2004; von Schassen, 2006) and the identified downstream targets of E2-induced αCaMKII signaling, ERK1/2, CREB, and MAP2, also play varied roles in this process (Sanchez, 2000; Cheng, 2002; Gerecke, 2004). Therefore, the effect of E2-induced αCaMKII signaling on the neurite outgrowth of primary hippocampal neurons was examined (Fig. 13A). A number of time points were investigated (data not shown) and 48 hr was chosen as the optimum time point to examine. The table shown in Figure 13B represents the compiled data for a variety of

measures of neurite outgrowth including mean neurite length, mean number of primary processes, mean branches, and neurite straightness. Specifically, E2 stimulation for 48 hr results in a significant increase in mean neurite length (73.91um ± 21.71 to 147.32um ± 20.97) as well as the mean number of primary processes extending from the soma (2.89 ± 0.39 to 4.98 ± 0.26) when compared to vehicle-stimulated neurons. There is a positive effect of E2 treatment on the number of branches as well; however, the increase compared to vehicle-treated is not statistically significant (2.40 ± 0.84 to 4.02 ± 0.95), but indicative of an effect nonetheless. Importantly, blocking CaMKII activity with KN-62 significantly inhibits the ability of E2 to increase these features of neurite outgrowth; mean outgrowth length is decreased to 65.05um±19.90um, and both the number of primary processes and branches are reduced as well to 2.92 ± 0.70 and 1.24 ± 0.60, respectively. The straightness of the processes, however, is unaffected by E2 treatment, and the use of KN-62 alone has no significant effect on neurite outgrowth (data not shown). Overall, these data indicate that E2-induced αCaMKII activation positively influences the neurite outgrowth of cultured primary hippocampal neurons.

Key Research Accomplishments:

- *In vivo* studies:
    - E2 but not vehicle administration induces ERK1/2 phosphorylation in whole tissue extracts of the rat uterine horn and brain.
    - E2 and PPT but not vehicle administration induces αCaMKII autophosphorylation in whole rat brain extracts
    - E2 and PPT but not vehicle administration enhances αCaMKII autophosphorylation in the rat hippocampus and dentate gyrus.
- αCaMKII is identified as a mediator in E2-induced ERK1/2 phosphorylation
- E2 treatment significantly and rapidly induces αCaMKII autophosphorylation NLT GnRH neurons, co-transfected Cos7 cells, and cultured embryonic hippocampal neurons
    - The E2-stimulated autophosphorylation is dependent on CaM action as well as Ca^{2+}-influx through L-type voltage-gated channels
- ERα and not ERβ is responsible for E2-induces αCaMKII/ERK1/2 activity
- ERα interacts with αCaMKII in a hormone-dependent manner to attenuate E2-induced αCaMKII autophosphorylation
- Pharmacological and RNAi technology show that E2-stimulated αCaMKII autophosphorylation results in ERK1/2 activity, which subsequently mediates CREB and MAP2 phosphorylation.
    - This signaling is dependent upon CaM action and Ca^{2+}-influx through L-type voltage-gated channels in NLT cells and primary hippocampal neurons
- E2-induced αCaMKII signaling positively influences the neurite outgrowth of primary hippocampal neurons
    - The mean length of neurites, the mean number of primary processes, and the mean number of branches are all increased

Reportable Outcomes:

Publications:
O'Neill EE, Blewett AR, Loria PM, Greene GL. The modulation of αCaMKII signaling by rapid ERα action. (under revision, **Brain Research**)

Recent Presentations:
Oral Presentations:
O'Neill EE, Blewett, AR, Greene GL
The Endocrine Society Meeting
Toronto, Canada – June 2007

Poster Presentations:
O'Neill EE, Blewett AR, Greene GL
Biomedical Sciences Retreat, University of Chicago
Lake Lawn Resort. Lake Delavan, WI - April 2007

O'Neill EE, Blewett AR, Greene GL
Biomedical Sciences Retreat, University of Chicago
Grand Geneva Resort and Spa. Lake Geneva, WI - May 2006

O'Neill EE Blewett AR, Loria PM, Greene GL
Keystone Symposia, Nuclear Receptors: Steroid Sisters Meeting
Fairmont Banff Springs. Banff, Alberta, Canada – March 2006

Conclusions:

The ability of E2 to influence signaling pathways in the brain has been examined with increasing interest however its effect on αCaMKII activity has not been described in detail. Our findings suggest a novel model for the activation of ERK1/2 by ERα via αCaMKII signaling. We have investigated the relationship between ER and CaMKII signaling in more detail as our previous data demonstrated that it was an important upstream regulator of ERK1/2 phosphorylation by E2. With this project we have provided evidence that E2 rapidly induces αCaMKII autophosphorylation in an ERα- and Ca^{2+} influx-dependent manner. This signaling is extranuclear and results in the phosphorylation of ERK1/2, CREB, and MAP2, and ultimately influences neurite outgrowth of embryonic primary hippocampal neurons. The association of ERα with αCaMKII negatively impacts the ability of E2 to induce αCaMKII autophosphorylation, suggesting a novel model for the modulation of αCaMKII activity by ERα.

Numerous studies indicate that E2's rapid effects in the brain are initiated outside of the nucleus (Kuroki, 2000; Mannella, 2006). Our ERα localization data in NLT neurons corresponds with other groups in that "classical" ERs are positioned not only in the nucleus, but in the cytoplasm and outgrowths as well (Clarke, 2000; Hart, 2007) where they are available to interact with cytoplasmic signaling cascades. Immunofluorescence and cell fractionation showed that E2-induced αCaMKII autophosphorylation in NLT neurons occurs specifically in the cell body and processes,

suggesting that the extranuclear pool of ER is responsible for mediating this event. ER action is clearly required as ICI treatment significantly blocks E2-induced αCaMKII autophosphorylation.

Based on our data, ERα and not ERβ mediates E2-induced αCaMKII autophosphorylation in the neuronal cell lines examined as well as in co-transfected Cos7 cells. These findings support the notion that the two subtypes do not have overlapping roles in brain function, which is not surprising because each has a distinct spatial and temporal expression patterns in the forebrain (Gonzalez, 2007) and the subtypes play distinct roles in neuronal function. For example, ERα mediates the neuroprotective effects of E2 against ischemic brain injury (Dubal, 2001) whereas ERβ is responsible for the anti-anxiety and anti-depressive effects of E2 (Walf, 2007). However, it has been reported that ERβ is involved in CA1 LTP and hippocampal-dependent contextual fear conditioning (Day, 2005), so a closer examination of ERβ-selective agonists in the hippocampus is needed to fully appreciate the role of ERβ in the activation of αCaMKII within this brain region.

An understanding of the mechanism underlying E2-induced αCaMKII activity is of great interest, and the simplest explanation is that E2 stimulates an increase in intracellular Ca^{2+} that subsequently activates αCaMKII since the effect of E2 on Ca^{2+} is well documented; E2 stimulates Ca^{2+} influx (Wu, 2005; Zhao, 2005) as well as the release of Ca^{2+} from intracellular stores (Beyer, 1998). Our data indicates that Ca^{2+} influx via L-type Ca^{2+} channels and CaM action are required for αCaMKII autophosphorylation by E2. However, the hormone-dependent association of ERα and αCaMKII adds a level of complexity to the signaling. The hypothesis that the ERα-αCaMKII interaction directly activates αCaMKII was proven incorrect. Instead, their interaction decreases the ability of E2 to activate αCaMKII by half; therefore, even though ERα is initially required for E2 to evoke αCaMKII activity via Ca^{2+} influx, it simultaneously downregulates the activity. These findings are in alignment with studies that examined a transgenic mouse model in which a constitutively active αCaMKII, CaMKII-Asp^{286}, is expressed in an inducible and forebrain-specific fashion (Mayford, 1996; Bejar, 2002; Yasuda, 2006). These studies found that indiscriminate CaMKII activity results in the loss of low-frequency-induced LTP and deficits in spatial memory. Bejar (2002) found that high expression of the transgene also affected fear-conditioned memory. Therefore, it is reasonable to imagine that ERα plays both a positive and negative role in E2-induced αCaMKII activation to prevent molecular and behavioral memory impairments. An alternative explanation is that the opposing effects of ER actually "primes" αCaMKII; it prevents αCaMKII from ever reaching maximal activity by E2, and instead lowers the threshold for subsequent Ca^{2+} signals, allowing for more efficient and perhaps, prolonged responses. Also, by maintaining sub-maximal activity, the dual role of ERα permits αCaMKII to function as a Ca^{2+} frequency detector to translate the information encoded by the Ca^{2+} spikes into various levels of kinase activity that correspond with specific cellular responses. Thus, the complex role played by ERα appears to be in place to maintain the sensitivity and selectivity of αCaMKII activity in neurons.

ERα is expressed in the hippocampus of rodents and humans (Solum, 2001; Adams, 2002; Hu, 2003), and there are numerous reports detailing the effects of E2 on the rodent hippocampus. For example, E2 increases dendritic spine density as well as

synapse density (Woolley, 1998), and influences membrane excitability and LTP of CA1, CA3, and dentate gyrus neurons (Woolley, 2007). Since αCaMKII is abundant in the hippocampus and its activity is essential certain memory processes (Silva, 1992), our finding that E2 and PPT enhance αCaMKII autophosphorylation in the hippocampus indicates that this signaling is physiologically relevant and potentially impacts neuronal plasticity *in vivo*. The activation is most dramatic in the dentate gyrus after 1 hr or 24 hr exposure. Interestingly, the dentate gyrus is one of the rare brain regions where adult neurogenesis occurs, which is thought to be crucial for the formation of new memories and clearance of unnecessary ones (Eriksson PS, 1998; Aimone JB, 2006). A closer examination of E2-induced αCaMKII activity in this region is necessary as it is enticing to hypothesize that E2 may affect memory by stimulating neurogenesis.

αCaMKII phosphorylates a wide variety of substrates involved in numerous neuronal processes (McGlade-McCulloh, 1993; Omkumar, 1996; Stefani, 2005; Liu, 2005). We have identified ERK1/2, CREB, and MAP2 as proteins targeted by E2-induced αCaMKII autophosphorylation. Like αCaMKII, all three are involved in neuronal differentiation and neurite outgrowth, and ERK1/2 and CREB have also been shown to play a role in LTP and memory processes (Trifilieff, 2006). E2 action has previously been linked to ERK1/2 and CREB phosphorylation in various populations of neurons and *in vivo* (Lee, 2004; Bryant, 2005; Szego, 2006), and we have provided evidence that αCaMKII action mediates their phosphorylation stimulated by E2 in NLT neurons and primary hippocampal neurons. CREB can be directly phosphorylated by αCaMKII (Wu, 2001), although in our studies it appears that CREB is indirectly affected by αCaMKII-mediated ERK1/2 activity since its phosphorylation by E2 was blocked by U0126 pre-treatment. This finding corresponds with reports demonstrating that CREB is phosphorylated by ribosomal S6 kinase (RSK) family members, which are first activated by ERK1/2 (Cammarota, 2001). Additionally, it is important to note that CaMKII has been shown to be upstream of ERK1/2 activity by several groups, even though the kinase cannot directly phosphorylate ERK1/2 (Franklin, 2000; Choe, 2001; Borbiev, 2003; Browning, 2005; Illario, 2005). Instead, it has been postulated that CaMKII activity influences the activation of a nonreceptor tyrosine kinase, Pyk2 (proline-rich tyrosine kinase 2), which subsequently activates c-Src and the rest of the MAPK cascade, ultimately resulting in ERK1/2 phosphorylation (Zwick, 1999; Ginnan, 2002). Our studies did not rigorously test this idea however we did generate preliminary data demonstrating that the inhibition of Pyk2 with salicylate disrupted the ability of E2 to induce the phosphorylation of ERK1/2 without affecting αCaMKII activity (data not shown). These results must be validated in order to begin to understand the mechanism by which CaMKII leads to ERK1/2 activity, although it is clear that CaMKII is involved in E2-stimulated ERK1/2 activity in NLT cells and primary hippocampal neurons since its inhibition by either KN-62 or siRNA significantly blocked ERK1/2 phosphorylation by E2 (Figs. 8A,10A,11). Furthermore, E2-induced ERK1/2 phosphorylation was also completely abolished by the inhibition of Ca^{2+} influx and CaM action (Figs. 8B and 10B), both of which are upstream of CaMKII activity. To the best of our knowledge we are the first to report that E2 is capable of stimulating MAP2 phosphorylation in primary hippocampal neurons. MAP2 is a direct target of αCaMKII, although it can also be phosphorylated by ERK1/2 (Sanchez, 2000) as we have confirmed in our studies; its phosphorylation by E2 is blocked by U0126 pre-treatment. Expectedly, both Ca^{2+}-influx

and CaM signaling are required for E2-induced ERK1/2, CREB, and MAP2 phosphorylation.

The use of pharmacological inhibitors and RNAi technology allowed us to order the observed E2-induced signaling (Fig. 14). ERα is rapidly activated by E2, which elevates intracellular Ca^{2+} levels by Ca^{2+} influx. αCaMKII detects the Ca^{2+} spike and undergoes autophosphorylation to become transiently active and mediate ERK1/2 activation. ERK1/2 subsequently leads to the phosphorylation of CREB and MAP2. However, the interaction of ERα with αCaMKII decreases E2-induced kinase autophosphorylation even though ER is initially required for this event via Ca^{2+} influx. It appears as though the activating signal of E2 dominates the negative effect of ER since there is a clear, positive downstream response to E2-activated αCaMKII, namely, neurite outgrowth. E2 increases mean neurite length, the number of primary processes per neuron, and the number of neurite branches of primary hippocampal neurons, as well as provided new evidence that αCaMKII is an essential mediator in this process. Neurite outgrowth is vital for neuronal communication and synaptic transmission, so it is noteworthy that E2-induced αCaMKII signaling positively influences it.

In summary, E2 rapidly evokes the ERα-dependent autophosphorylation of αCaMKII in immortalized GnRH neurons, primary hippocampal neurons, and the rat hippocampus *in vivo*, with a dramatic effect in the dentate gyrus. The activity requires Ca^{2+} influx and CaM action, and is kept in check by the interaction of ERα with αCaMKII, which potentially maintains the sensitivity and selectivity of αCaMKII activity. Ultimately, E2-stimulated αCaMKII activity results in the phosphorylation of ERK1/2, CREB, and MAP2, and enhanced neurite outgrowth of primary hippocampal neurons.

There are larger implications for this study beyond the description of a novel signaling pathway. Given the dramatic increase in female life expectancy in industrialized nations during the last century, from 54 years old in 1900 to 83 years old today, and the fact that the onset of menopause has remained stable at approximately 50 years of age since recorded history (Sherwin, 2003), women now live more than one third of their lives in an estrogen-deprived state. It has been shown by numerous studies that estrogen deprivation leads not only to deficits in various aspects of brain function, including cognition, but can increase the risk of developing osteoporosis as well as certain cardiovascular syndromes such as artherosclerosis. Unfortunately, many women opt out of receiving hormone replacement therapy even after understanding its numerous benefits for fear of increasing their risk of developing breast cancer. Therefore, novel hormone therapies that maintain the estrogenic benefits in nonreproductive tissues but remain inactive in the breast need to be explored. Our characterization of E2-induced αCaMKII signaling pathway can be exploited to create new SERMs that can potentially enhance memory and cognitive function in postmenopausal women without affecting the breast tissue or increasing the risk of developing breast cancer.

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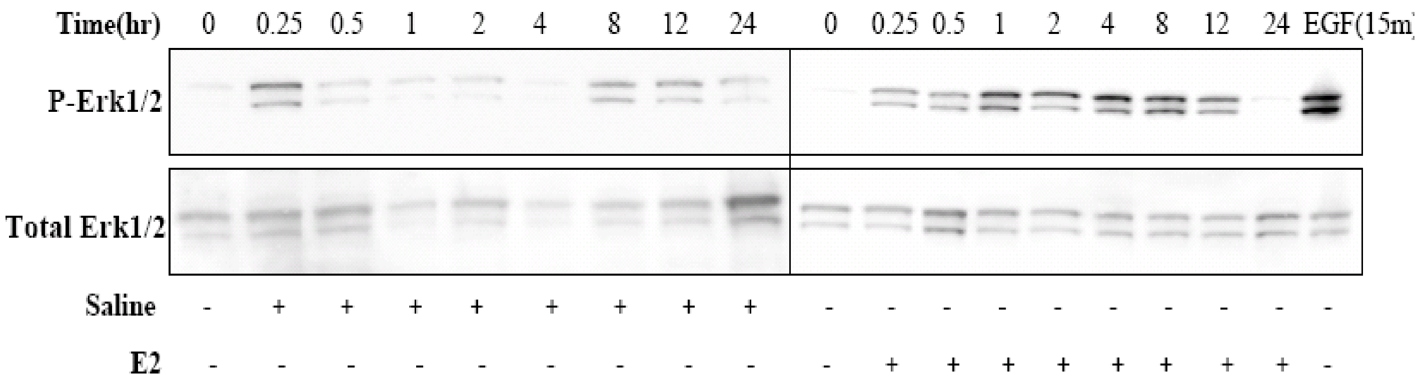
Appendix 1: Supporting Data

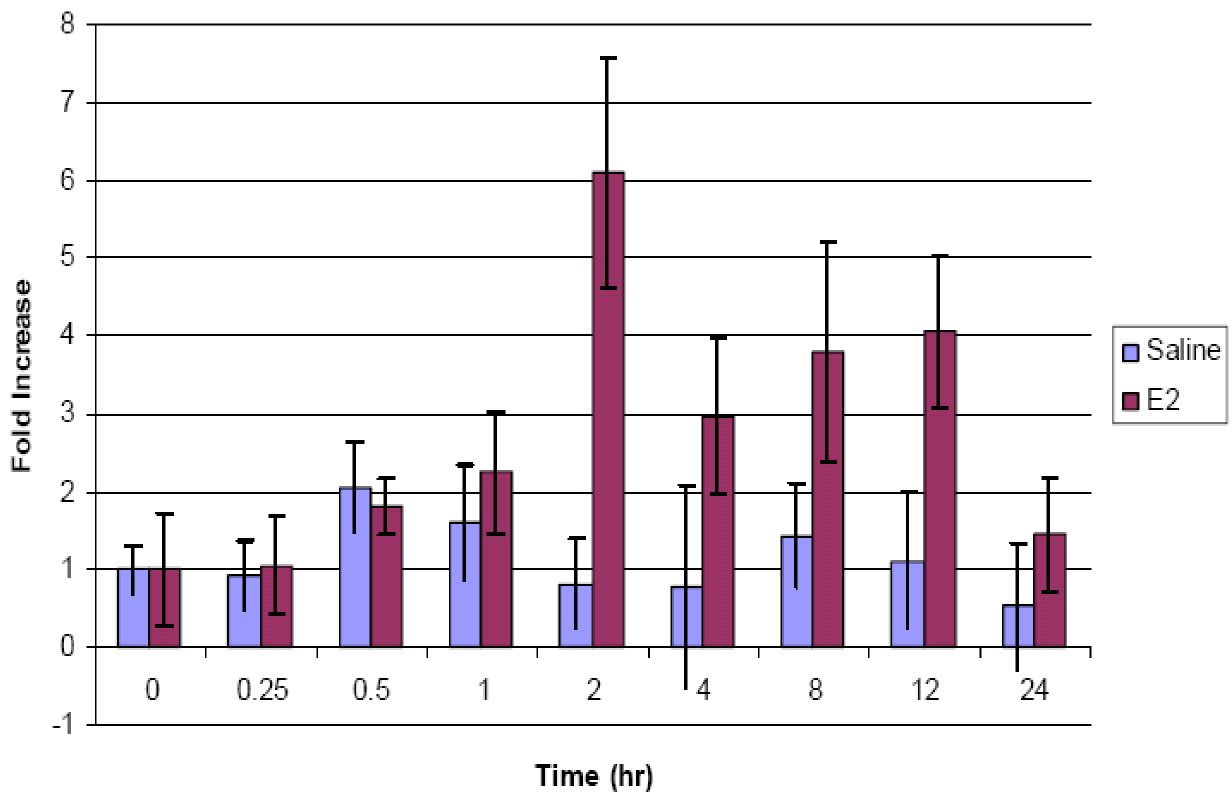
Figure 1

a.



Time(hr) 0 0.25 0.5 1 2 4 8 12 24 0 0.25 0.5 1 2 4 8 12 24 EGF(15m)
P-Erk1/2
Total Erk1/2
Saline - + + + + + + + + - - - - - - - - - -
E2 - - - - - - - - - - + + + + + + + + -

b.



Fold Increase
8
7
6
5
4
3
2
1
0
-1
0
0.25
0.5
1
2
4
8
12
24
Time (hr)
Saline
E2

Figure 1. Estrogen stimulates ERK1/2 activation in rat uterine horns. a) ovariectomized female rats (21 days old) were intraperitoneally injected with either saline control or E2(0.1ug) or EGF (0.1ug) for the time period indicated. The uterine horns were then removed,homogenized, and western blot analysis was performed to detect Erk-1 and -2 phosphorylation relative to total Erk-1 and -2 expression. Representative of 6 replicates. b) Graphic representation of all 6 replicates.

Figure 2

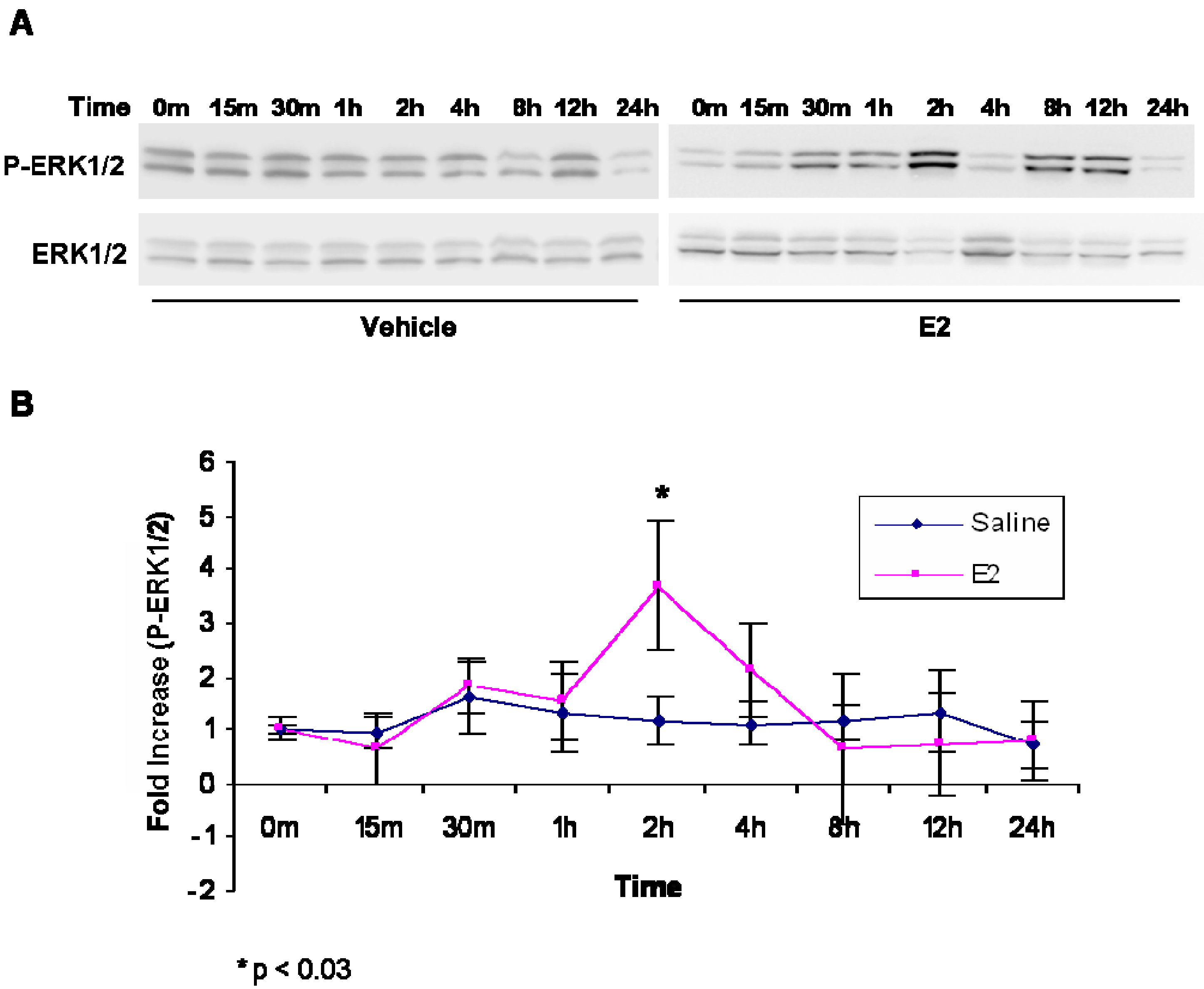
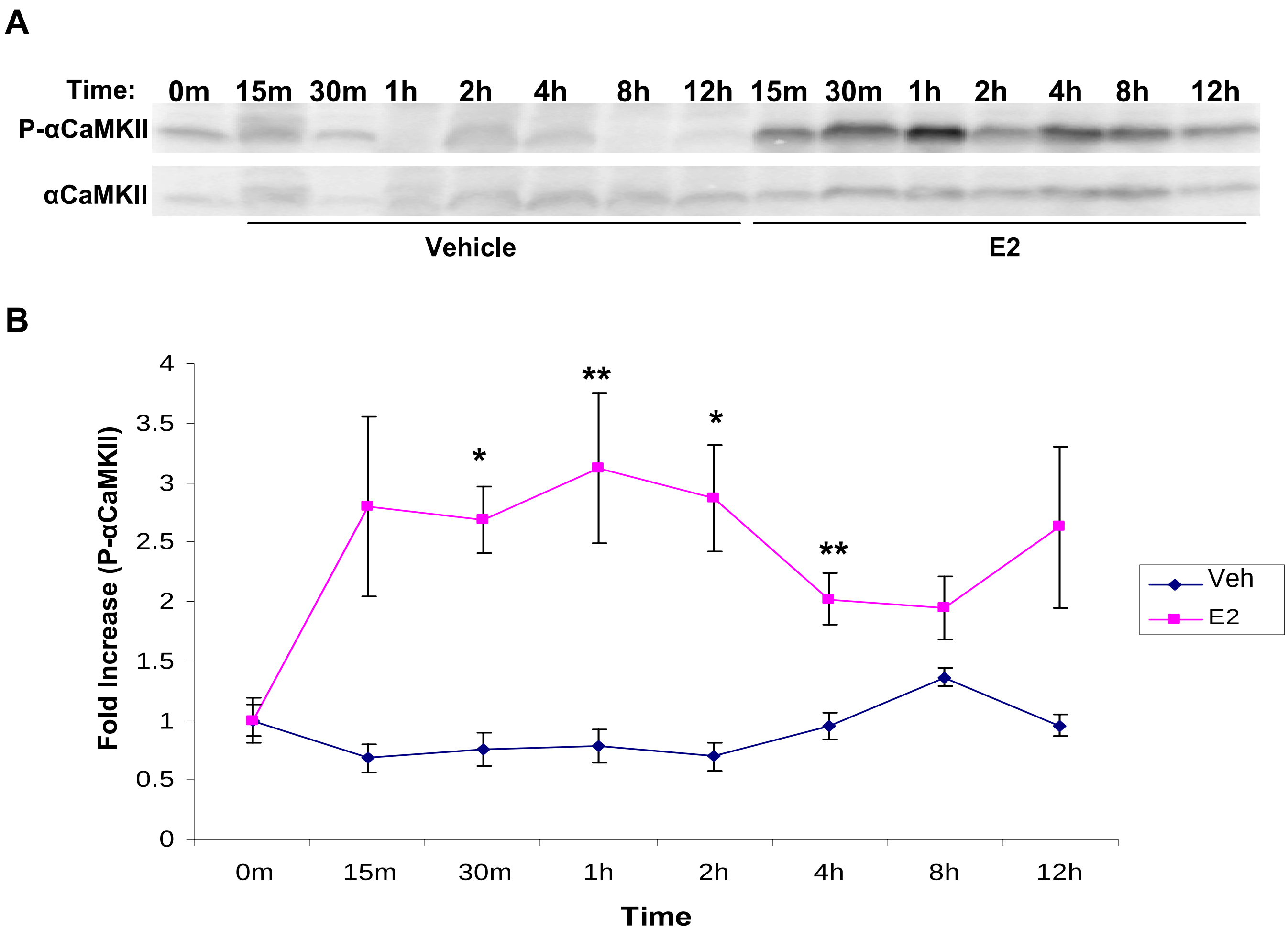

A
Time
0m 15m 30m 1h 2h 4h 8h 12h 24h
0m 15m 30m 1h 2h 4h 8h 12h 24h
P-ERK1/2
ERK1/2
Vehicle
E2
B
Fold Increase (P-ERK1/2)
6
5
4
3
2
1
0
-1
-2
*
Saline
E2
0m 15m 30m 1h 2h 4h 8h 12h 24h
Time
* p < 0.03

Figure 2. E2 stimulates ERK1/2 activation in rat brain. a) ovariectomized female rats (21 days old) were intraperitoneally injected with either saline control or E2(0.1ug) for the time periods indicated. The brain was then removed, homogenized, and western blot analysis was performed to detect ERK1/2 phosphorylation relative to total ERK1/2 expression. b) Graphic representation of all 6 replicates.

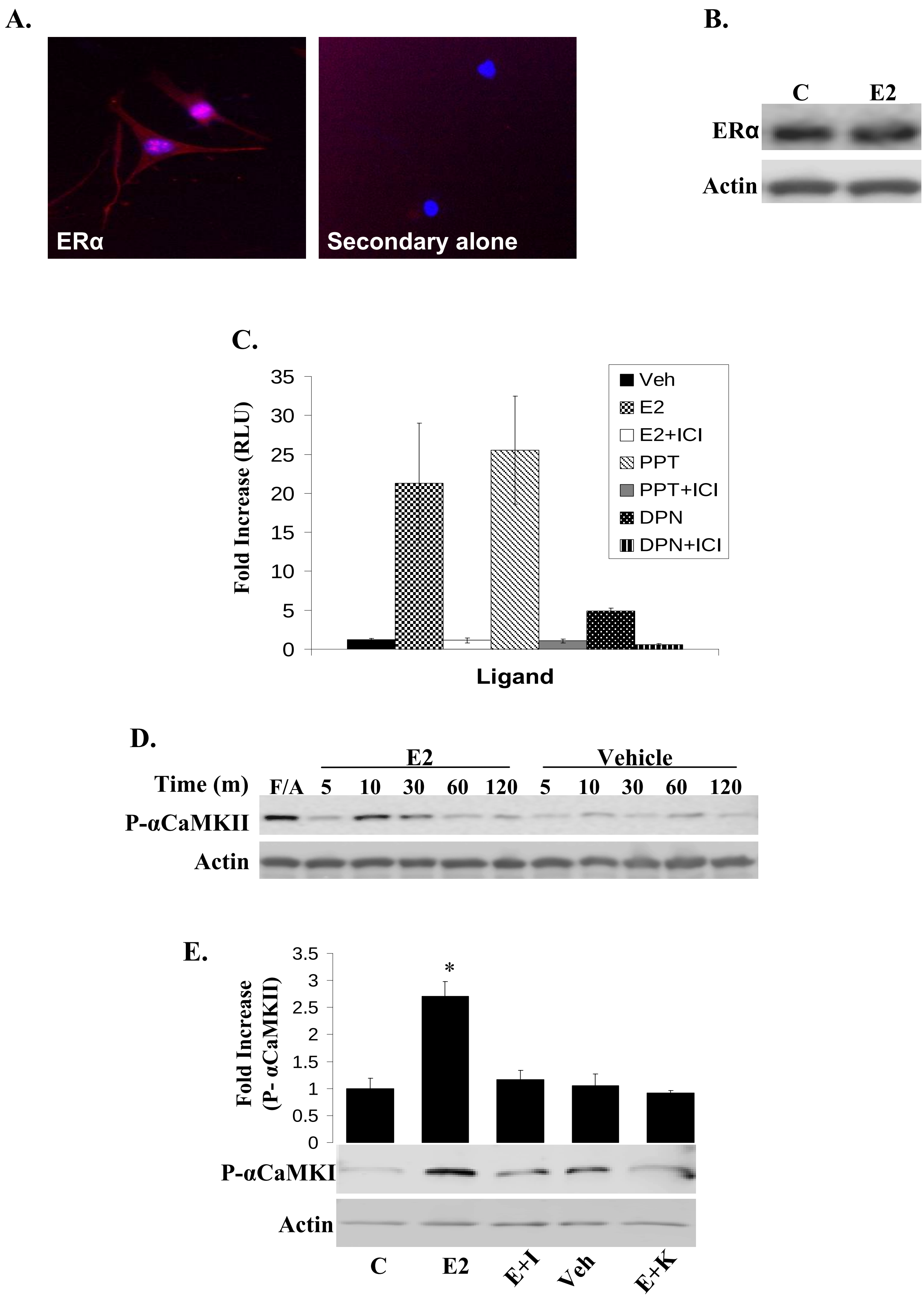
Figure 3



* p < 0.01, ** p < 0.05

Figure 3. Systemic administration of E2 to ovariectomized rats stimulates αCaMKII autophosphorylation in whole brain extract. **(A)** Ovariectomized 21 day old female Sprague Dawley rats were injected intraperitoneally with vehicle (sterile saline) or E2 (5ug/kg) for the times indicated. Brain tissue was harvested, pulverized, and immunoblotting on the resulting homogenate was used to detect total and autophosphorylated αCaMKII. Representative blot. **(B)** Density values for phosphorylated kinase was normalized to total kinase levels for 6 rats/condition and presented as the mean fold increase over unstimulated control ± S.E.M.

Figure 4



A.
ERα
Secondary alone
B.
C
E2
ERα
Actin
C.
Fold Increase (RLU)
35
30
25
20
15
10
5
0
Ligand
Veh
E2
E2+ICI
PPT
PPT+ICI
DPN
DPN+ICI
D.
E2
Vehicle
Time (m)
F/A
5
10
30
60
120
5
10
30
60
120
P-αCaMKII
Actin
E.
Fold Increase (P- αCaMKII)
3.5
3
2.5
2
1.5
1
0.5
0
*
P-αCaMKI
Actin
C
E2
E+I
Veh
E+K

Figure 4. NLT cells express functional ERα and ERβ and E2 rapidly induces the autophosphorylation of αCaMKII. **(A)** Unstimulated NLT cells were co-stained for ERα (red) and DAPI (blue). Left panel: immunofluorescence performed with primary antibody (MC-20). Right panel: immunofluorescence performed in the absence of primary antibody **(B)** NLT cells were either unstimulated (C) or treated for 10 min with E2 (10nM), and immunoblotting detected total ERα and pan-Actin. **(C)** NLT cells were transfected with 3ERE-Luc and β-galactosidase, treated with vehicle (Veh; 0.01% EtOH), E2 (10nM), E2+ICI (10nM, 1uM), PPT (100nM), PPT+ICI (100nM, 1uM), DPN (100nM), or DPN+ICI (100nM, 1uM) for 24 hours, and assayed for both luciferase and β-galactosidase activity. Luciferase activity was normalized for transfection using β-galactosidase and represented as fold increase RLU (relative light units) over vehicle-treated cells. **(D)** NLT cells were treated with forskolin and A23187 (F/A) for 2 min and 20 min, respectively, as a positive control, or with E2 (10nM) or vehicle (0.01% EtOH) for the indicated times. Immunoblotting detected autophosphorylated αCaMKII and pan-Actin. **(E)** NLT cells were either unstimulated (C) or treated with vehicle (Veh; 0.01% EtOH), E2 (10nM), E2+ICI (E+I; 10nM, 1uM), or E2+KN-62 (E+K; 10nM, 10uM) for 10 min. ICI and KN-62 were applied 30 min prior to E2 treatment. Immunoblotting detected autophosphorylated αCaMKII and pan-Actin. Representative blot. Densitometry for phosphorylated kinase was normalized to total protein levels for 3 independent experiments, and presented as the mean fold increase over unstimulated control ± S.D. (*) $p < 0.01$, E2 relative to other treatment conditions.

Figure 5

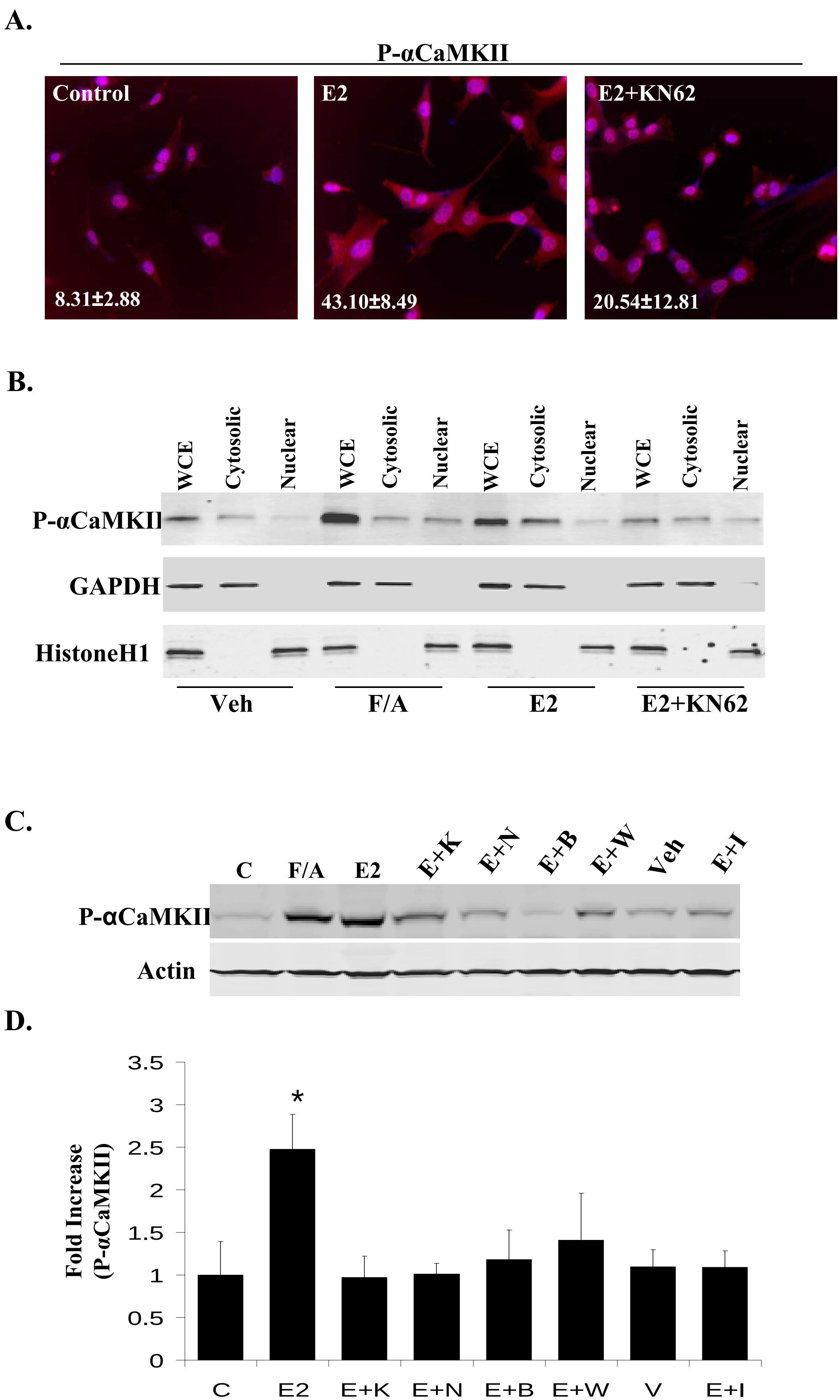
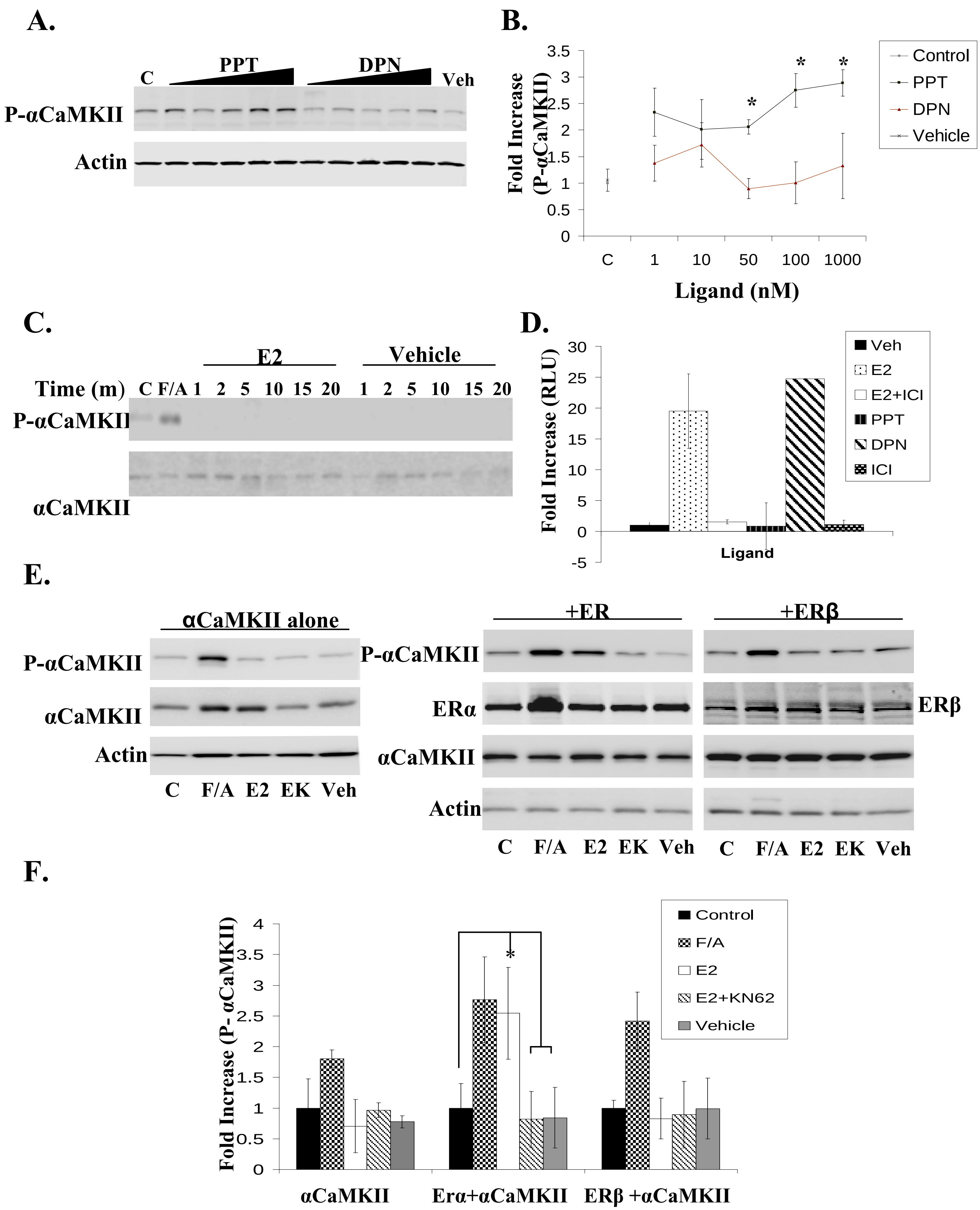

A.
P-αCaMKII
Control
E2
E2+KN62
8.31±2.88
43.10±8.49
20.54±12.81
B.
WCE
Cytosolic
Nuclear
WCE
Cytosolic
Nuclear
WCE
Cytosolic
Nuclear
WCE
Cytosolic
Nuclear
P-αCaMKII
GAPDH
HistoneH1
Veh
F/A
E2
E2+KN62
C.
C
F/A
E2
E+K
E+N
E+B
E+W
Veh
E+I
P-αCaMKII
Actin
D.
Fold Increase (P-αCaMKII)
3.5
3
2.5
2
1.5
1
0.5
0
*
C
E2
E+K
E+N
E+B
E+W
V
E+I

Figure 5. E2-induced αCaMKII autophosphorylation occurs in the cytoplasm and cell extensions of NLT cells in a Ca2+/CaM-dependent manner. (A) Cells were either unstimulated (C) or treated with E2 (10nM) or E2+KN-62 pre-treatment (10nM, 10uM) for 10 min and then stained for autophosphorylated αCaMKII (red) and DAPI (blue) to visualize localization of kinase activation. **(B)** Whole cell extract (WCE), cytosolic, and nuclear fractions were obtained from NLT cells treated with vehicle (Veh; 0.01%EtOH), F/A (0.5mM, 50uM), E2 (10nM), or E2+KN-62 30 min pre-treatment (10nM, 10uM) and analyzed by immunoblotting for autophosphorylated αCaMKII, GAPDH (cytosolic protein), and Histone H1 (nuclear protein). **(C)** NLT cells were either unstimulated (C) or treated for 10 min with Veh (0.01%EtOH), F/A (0.5mM, 50uM), E2 (10nM), or E2 (10nM) with 30 min pre-treatment of KN-62 (E+K; 10uM), Nifedipine (E+Nif; 10uM), BAPTA-AM (E+B; 10uM), W7 (E+W; 10uM), or ICI (E+I; 1uM). Representative blot. **(D)** Autophosphorylated αCaMKII was normalized to total protein levels from 3 independent experiments and presented as the mean fold increase over unstimulated control ± S.D. (*) $p < 0.03$, E2 relative to other treatment conditions.

Figure 6



A.
C
PPT
DPN
Veh
P-αCaMKII
Actin
B.
Fold Increase (P-αCaMKII)
Control
PPT
DPN
Vehicle
C 1 10 50 100 1000
Ligand (nM)
C.
E2
Vehicle
Time (m) C F/A 1 2 5 10 15 20 1 2 5 10 15 20
P-αCaMKII
αCaMKII
D.
Fold Increase (RLU)
Veh
E2
E2+ICI
PPT
DPN
ICI
Ligand
E.
αCaMKII alone
+ER
+ERβ
P-αCaMKII
αCaMKII
Actin
ERα
ERβ
C F/A E2 EK Veh
F.
Fold Increase (P- αCaMKII)
Control
F/A
E2
E2+KN62
Vehicle
αCaMKII
Erα+αCaMKII
ERβ +αCaMKII

Figure 6. ERα and not ERβ mediates αCaMKII autophosphorylation. **(A)** NLT cells were either unstimulated (C) or treated with Veh (0.01%EtOH) or increasing concentrations of PPT or DPN (1nM-1000nM) for 10 min and autophosphorylated αCaMKII and pan-Actin levels were detected with western blot analysis. Representative blot. **(B)** Densitometry analysis for phosphorylated kinase was normalized to total protein levels from 3 independent experiments, and presented as the mean fold increase over unstimulated control ± S.D. (*) $p < 0.02$. **(C)** SK-N-SH cells were treated with Veh (0.01%EtOH), F/A (0.5mM, 50uM), or E2 (10nM) for the times indicated and immunoblotting was used to detect total and autophosphorylated αCaMKII. **(D)** SK-N-SH cells were transfected with 3ERE-Luc and β-galactosidase, treated with Veh (0.01% EtOH), E2 (10nM), E2+ICI (10nM, 1uM), PPT (100nM), DPN (100nM), or ICI (1uM) for 24 hours, and assayed for both luciferase and β-galactosidase activity. Luciferase activity was normalized for transfection using β-galactosidase and represented as fold increase RLU over vehicle-treated cells. **(E)** Cos7 cells co-transfected with αCaMKII and either ERα or ERβ were unstimulated (C) or treated with Veh (0.01%EtOH), F/A (0.5mM, 50uM), E2 (10nM), or E2+KN-62 (E+K; 10nM, 10uM) for 2 min and then subjected to immunoblotting to detect autophosphorylated αCaMKII, total αCaMKII, ERα or ERβ, and pan Actin. Representative blot. **(F)** Densitometry analysis for phosphorylated kinase was normalized to total protein levels for 3 independent experiments, and presented as the mean fold increase over unstimulated control ± S.D. (*) $p < 0.02$.

Figure 7

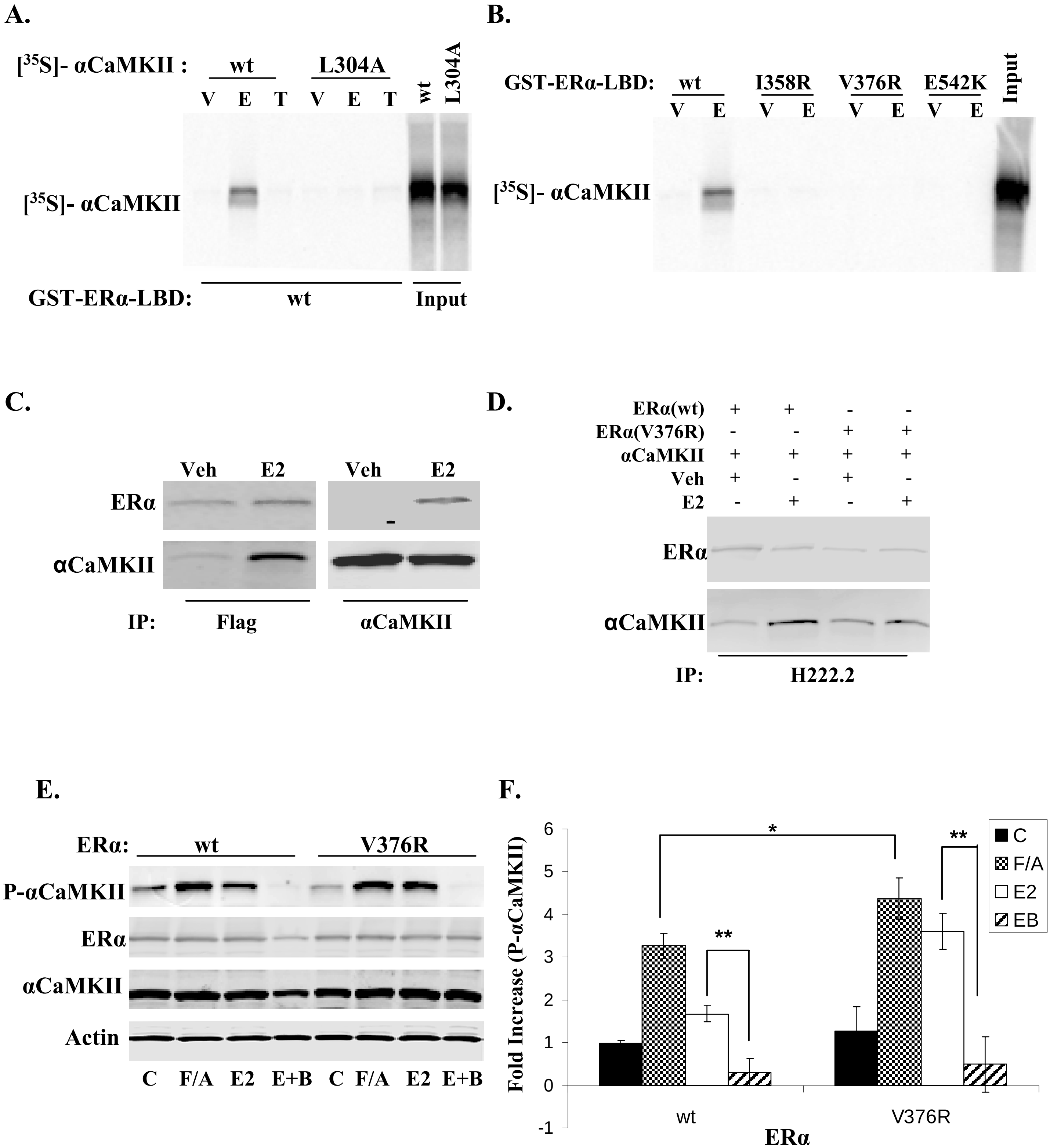
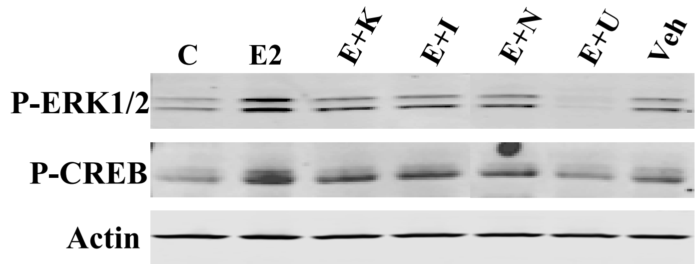

A.
[35S]- αCaMKII :
wt
L304A
V E T V E T wt L304A
[35S]- αCaMKII
GST-ERα-LBD:
wt
Input
B.
GST-ERα-LBD:
wt I358R V376R E542K Input
V E V E V E V E
[35S]- αCaMKII
C.
Veh E2 Veh E2
ERα
αCaMKII
IP: Flag αCaMKII
D.
ERα(wt) + + - -
ERα(V376R) - - + +
αCaMKII + + + +
Veh + - + -
E2 - + - +
ERα
αCaMKII
IP: H222.2
E.
ERα: wt V376R
P-αCaMKII
ERα
αCaMKII
Actin
C F/A E2 E+B C F/A E2 E+B
F.
Fold Increase (P-αCaMKII)
6
5
4
3
2
1
0
-1
*
**
**
C
F/A
E2
EB
wt
V376R
ERα

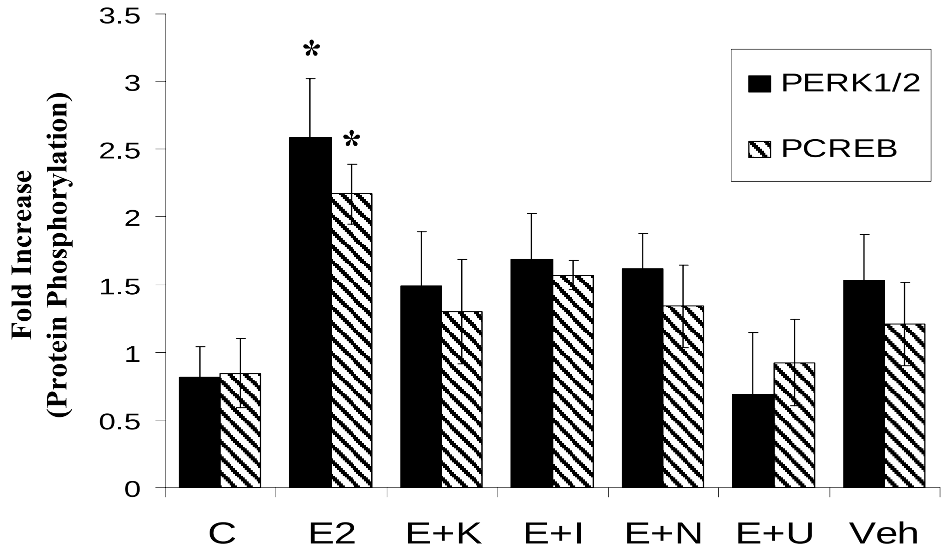
Figure 7. ERα interacts with αCaMKII in a hormone-dependent manner to attenuate E2-induced αCaMKII activity. (A) GST-ERα-LBD pulldown of [35S]-αCaMKII (wild-type and L304A). See materials and methods for details. V = vehicle, 1% EtOH; E = E2, 1uM; T = 4-hydroxy-tamoxifen, 1uM. Bound αCaMKII was visualized by autoradiography. **(B)** GST-ERα-LBD pulldown of wild-type [35S]-αCaMKII using wild type GST-ERα-LBD or GST-ERα-I358R, GST-ERα-V376R, or GST-ERα-E542K. V = vehicle, 1% EtOH; E = E2, 1uM. Bound αCaMKII was visualized by autoradiography. **(C)** Cos7 cells co-transfected with FLAG-ERα and αCaMKII and treated with Veh (0.01%EtOH) or E2 (10nM) for 10 min were immunoprecipitated with either anti-FLAG or anti-αCaMKII. Immunoblotting was used to detect ERα and αCaMKII. **(D)** Cos7 cells were co-transfected with αCaMKII and either wild type ERα or the interaction mutant ERα (V376R), treated with Veh (0.01%EtOH) or E2 (10nM) for 10 min were immunoprecipitated with either H222.2 or anti-αCaMKII. Immunoblotting was used to detect ERα and αCaMKII. **(E)** Cos7 cells were co-transfected with αCaMKII and either wild-type ERα or the interaction mutant ERα (V376R), treated with F/A (0.5mM, 50uM), E2 (10nM), E2+BAPTA-AM pre-treatment (E+B; 10nM, 10uM) for 2 min, or left unstimulated (C). Immunoblotting detected autophosphorylated αCaMKII, total αCaMKII, ERα, and pan-Actin. Representative blot. **(F)** Density values for autophosphorylated αCaMKII were normalized to total protein levels for 3 independent experiments, and presented as the mean fold increase over unstimulated control ± S.D. (*) $p < 0.03$, (**) $p < 0.02$.

Figure 8

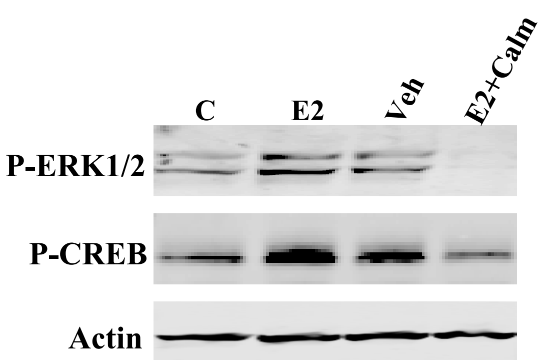
A.



B.



C.



D.

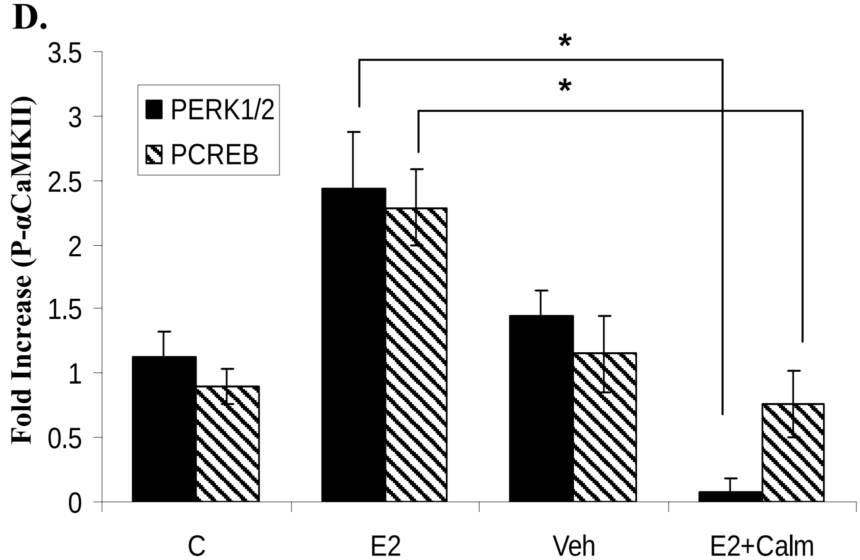


Figure 8. αCaMKII and Ca^{2+}/CaM signaling are involved in E2-induced ERK1/2 and CREB phosphorylation. (A) NLT cells were either unstimulated (C) or treated for 10 min with Veh (0.01% EtOH), E2 (10nM), or E2 (10nM) with 30 min pre-treatment of KN-62 (E+K; 10uM), ICI (E+I; 1uM), Nifedipine (E+N; 10uM), or U0126 (E+U; 10uM). Immunoblotting detected phosphorylated levels of ERK1/2 and CREB as well as pan-Actin. Representative blot. **(B)** Densitometry analysis for phosphorylated kinase was normalized to total protein levels for 3 independent experiments, and presented as the mean fold increase over unstimulated control ± S.D. (*) $p<0.03$, E2 compared to the rest of the conditions. **(C)** NLT cells were either unstimulated (C) or treated for 10 min with Veh (0.01% EtOH), E2 (10nM), or E2 (10nM) with 30 min pre-treatment of calmidazolium (E+Calm; 10uM). Immunoblotting detected phosphorylated ERK1/2 and CREB, and pan-Actin. Representative blot. **(D)** Densitometry for phosphorylated kinase was normalized to total protein levels, and presented as the mean fold increase over unstimulated control ± S.D. (*) $p < 0.02$.

Figure 9

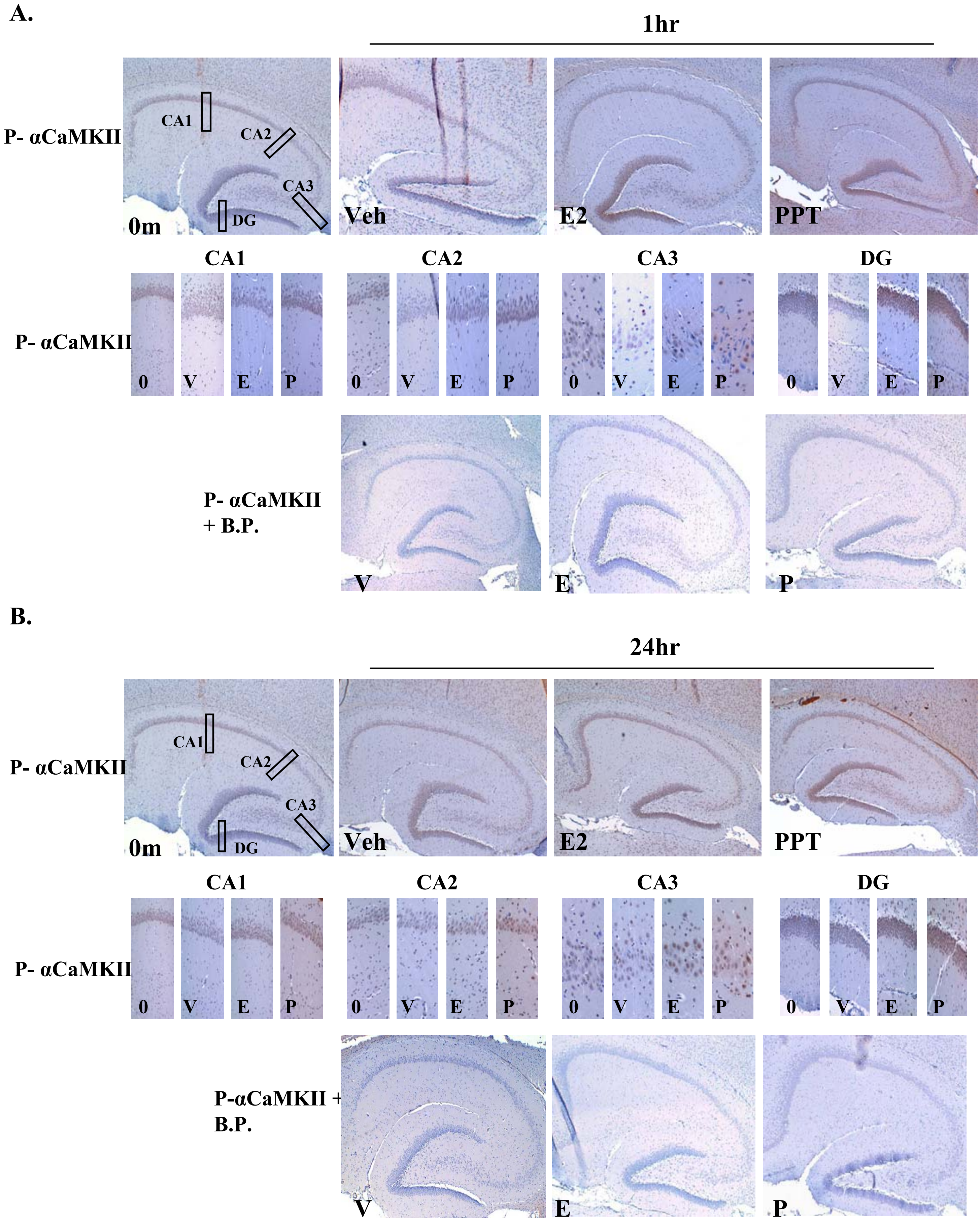

A.
1hr
P- αCaMKII
CA1
CA2
CA3
DG
0m
Veh
E2
PPT
CA1
CA2
CA3
DG
P- αCaMKII
0
V
E
P
P- αCaMKII
+ B.P.
V
E
P
B.
24hr
P- αCaMKII
CA1
CA2
CA3
DG
0m
Veh
E2
PPT
CA1
CA2
CA3
DG
P- αCaMKII
0
V
E
P
P-αCaMKII +
B.P.
V
E
P

Figure 9. E2 or PPT enhances αCaMKII autophosphorylation in the rat hippocampus *in vivo*. Ovariectomized 21 day old female Sprague Dawley rats were injected subcutaneously with vehicle (10% cremaphor/2% EtOH in saline), E2 (5ug/kg), or PPT (250ug/rat), and immunohistochemistry was performed on sagittal sections to detect autophosphorylated αCaMKII at **(A)** 1hr or **(B)** 24hr of ligand exposure. **Upper panels:** 4x representation of hippocampus. **Middle panels:** 20x representation of CA1, CA2, CA3 pyramidal neurons and the dentate gyrus (DG). **Lower panels:** IHC for autophosphorylated αCaMKII performed in the presence of a specific blocking peptide in 10x excess.

Figure 10

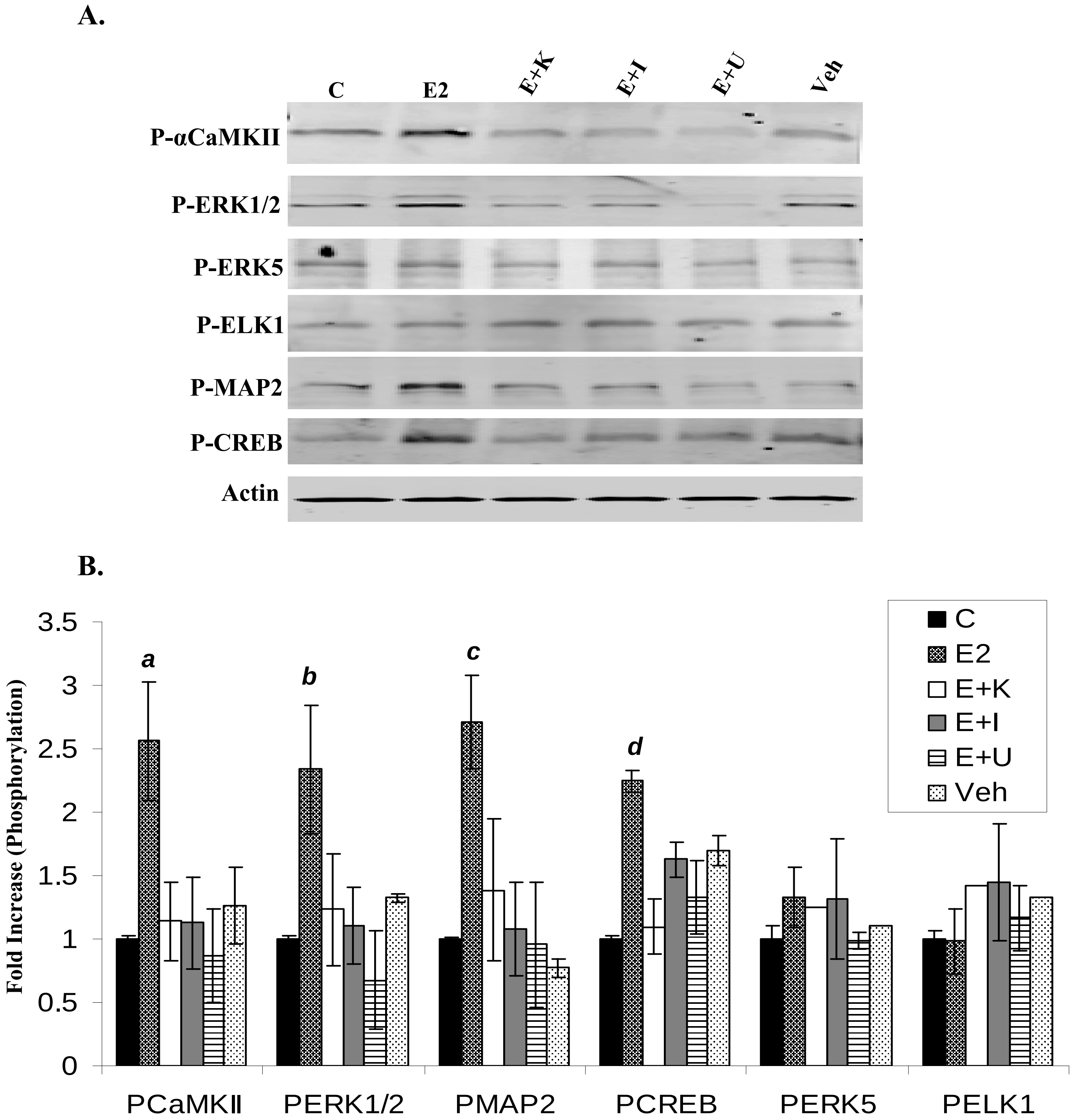

A.
C
E2
E+K
E+I
E+U
Veh
P-αCaMKII
P-ERK1/2
P-ERK5
P-ELK1
P-MAP2
P-CREB
Actin
B.
Fold Increase (Phosphorylation)
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
d
C
E2
E+K
E+I
E+U
Veh
PCaMKII
PERK1/2
PMAP2
PCREB
PERK5
PELK1

Figure 10. Rapid E2 action induces αCaMKII autophosphorylation as well as the phosphorylation of downstream proteins in a αCaMKII-dependent manner in primary hippocampal neurons. (A) Cultured embryonic primary hippocampal neurons were either unstimulated (C) or treated with Veh (0.01% EtOH), E2 (1nM), or E2 (1nM) with 30 min pre-treatment of KN-62 (E+K; 10uM), ICI (E+I; 1uM), or U0126 (E+U; 10uM). Immunoblotting detected phosphorylated levels of αCaMKII, ERK1/2, ERK5, ELK1, CREB, and MAP2 as well as pan-Actin. Representative blot. **(B)** Densitometry for phosphorylated kinase was normalized to total protein levels for 3 independent experiments, and presented as the mean fold increase over unstimulated control ± S.D. *a)* $p<0.02$; *b*) $p < 0.05$; *c)* $p<0.04$; *d)* $p<0.05$, comparing E2 treatment to other conditions.

Figure 11

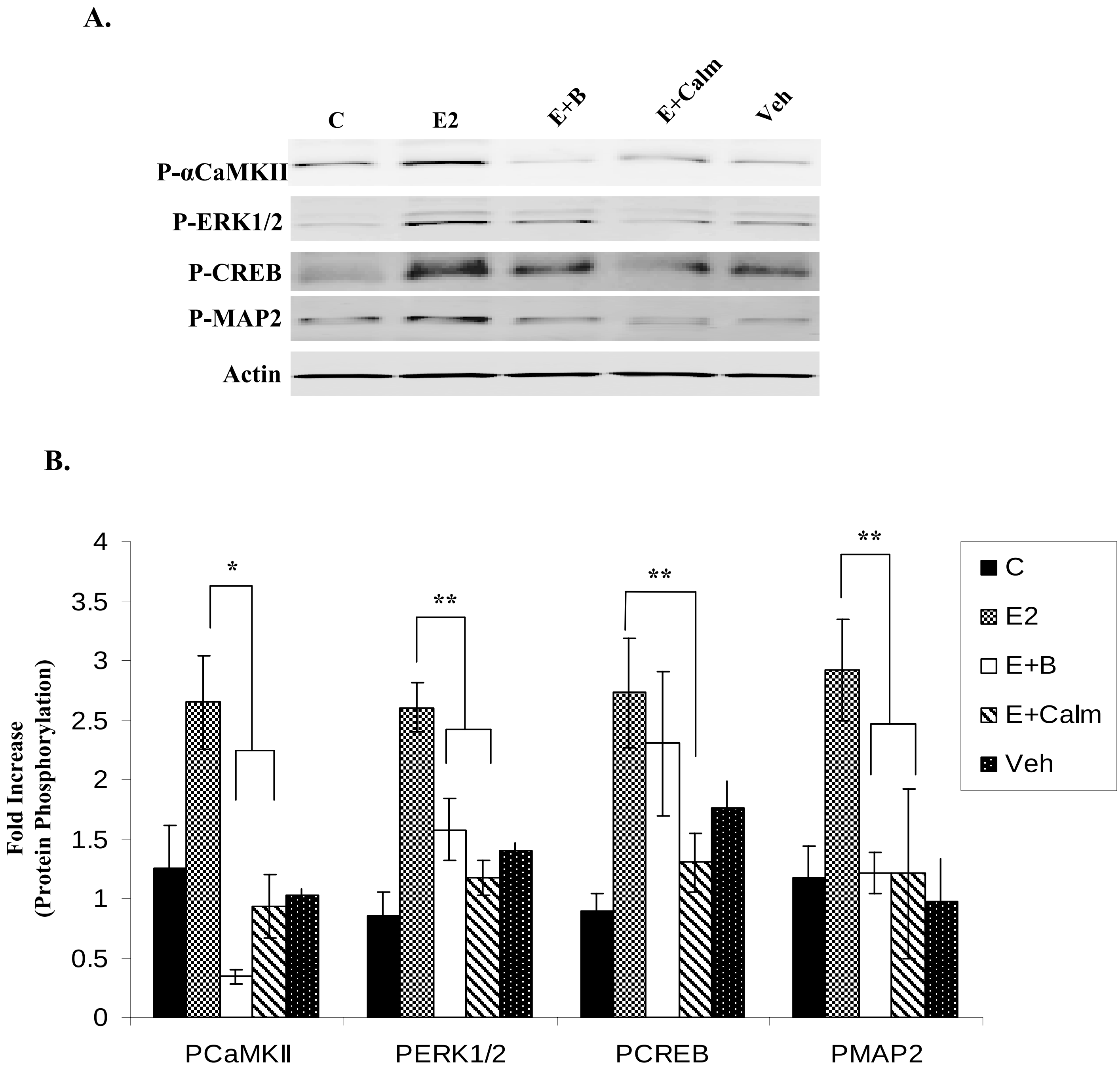

A.
C
E2
E+B
E+Calm
Veh
P-αCaMKII
P-ERK1/2
P-CREB
P-MAP2
Actin
B.
Fold Increase
(Protein Phosphorylation)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
**
**
**
PCaMKII
PERK1/2
PCREB
PMAP2
C
E2
E+B
E+Calm
Veh

Figure 11. Ca^{2+}/CaM action are required for E2-induced ERK1/2, CREB, and MAP phosphorylation in primary hippocampal neurons. (A) Cultured embryonic primary hippocampal neurons were either unstimulated (C) or treated with Veh (0.01% EtOH), E2 (1nM), or E2 (1nM) with 30 min pre-treatment of BAPTA-AM (E+B; 10uM) or calmidazolium (E+Calm; 10uM). Immunoblotting detected phosphorylated levels of αCaMKII, ERK1/2, CREB, and MAP2 as well as pan-Actin. Representative blot. **(B)** Densitometry for phosphorylated kinase was normalized to total protein levels, and presented as the mean fold increase over unstimulated control ± S.D. (*) $p < 0.01$, (**) $p < 0.02$.

Figure 12

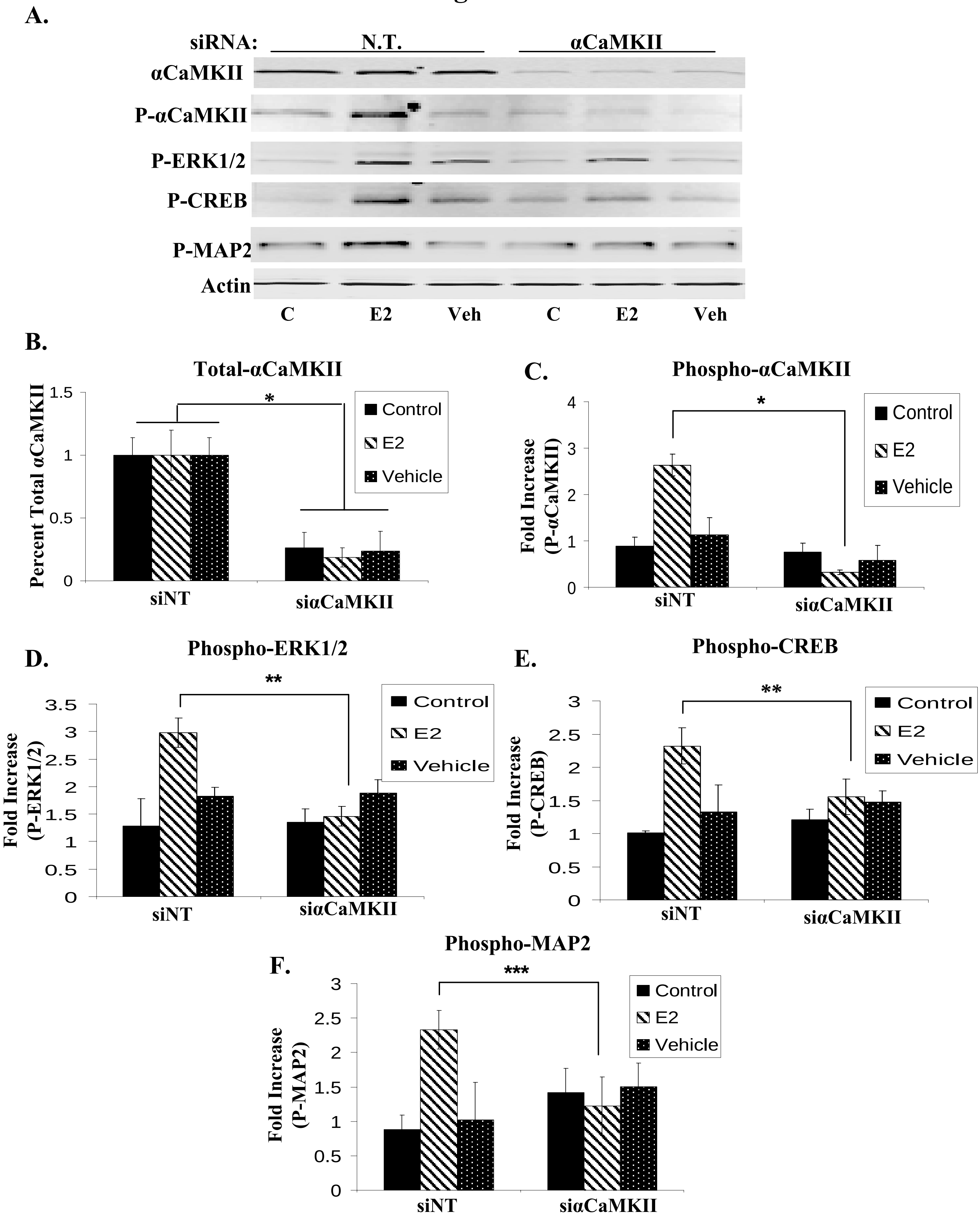
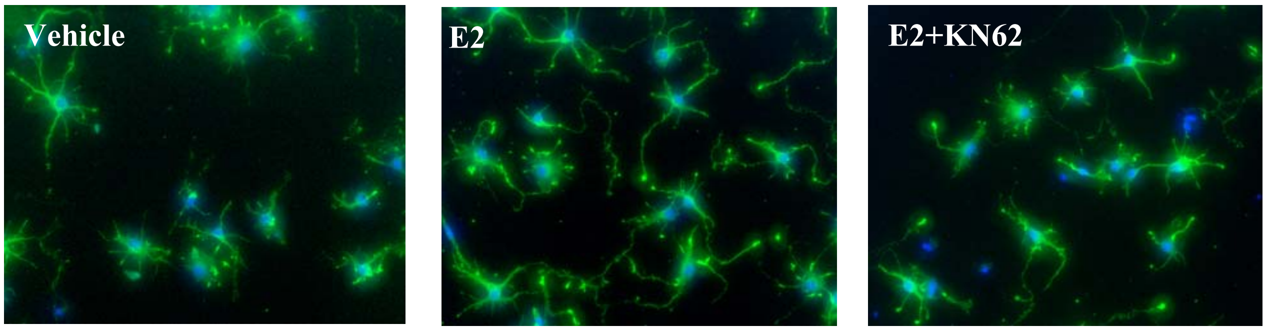

A.
siRNA:
N.T.
αCaMKII
αCaMKII
P-αCaMKII
P-ERK1/2
P-CREB
P-MAP2
Actin
C
E2
Veh
C
E2
Veh
B.
Total-αCaMKII
Percent Total αCaMKII
*
Control
E2
Vehicle
siNT
siαCaMKII
C.
Phospho-αCaMKII
Fold Increase (P-αCaMKII)
*
Control
E2
Vehicle
siNT
siαCaMKII
D.
Phospho-ERK1/2
Fold Increase (P-ERK1/2)
**
Control
E2
Vehicle
siNT
siαCaMKII
E.
Phospho-CREB
Fold Increase (P-CREB)
**
Control
E2
Vehicle
siNT
siαCaMKII
F.
Phospho-MAP2
Fold Increase (P-MAP2)
***
Control
E2
Vehicle
siNT
siαCaMKII

Figure 12. Targeted inhibition of αCaMKII blocks E2-induced ERK1/2, CREB, and MAP2 phosphorylation. (A) Cultured embryonic primary hippocampal neurons were transfected with either non-targeting siRNA (siN.T.)or siαCaMKII, and were unstimulated (C) or treated with Veh (0.01%EtOH) or E2 (1nM). Total αCaMKII, autophosphorylated αCaMKII, phosphorylated levels of ERK1/2, CREB, MAP2, and pan-Actin expression were then assayed via immunoblotting . Representative blot. **(B)** Densitometry for total αCaMKII was normalized to pan-Actin and presented as the mean relative protein expression ± S.D. Additionally, the phosphorylation status of αCaMKII **(C)**, ERK1/2 **(D)**, CREB **(E)**, and MAP2 **(F)** was examined post-siRNA introduction and ligand treatment. Phosphorylated levels of the indicated proteins were normalized to pan-Actin and presented as the mean fold increase over unstimulated control ± S.D. * $p < 0.01$; ** $p < 0.03$; *** $p < 0.05$.

Figure 13

A.



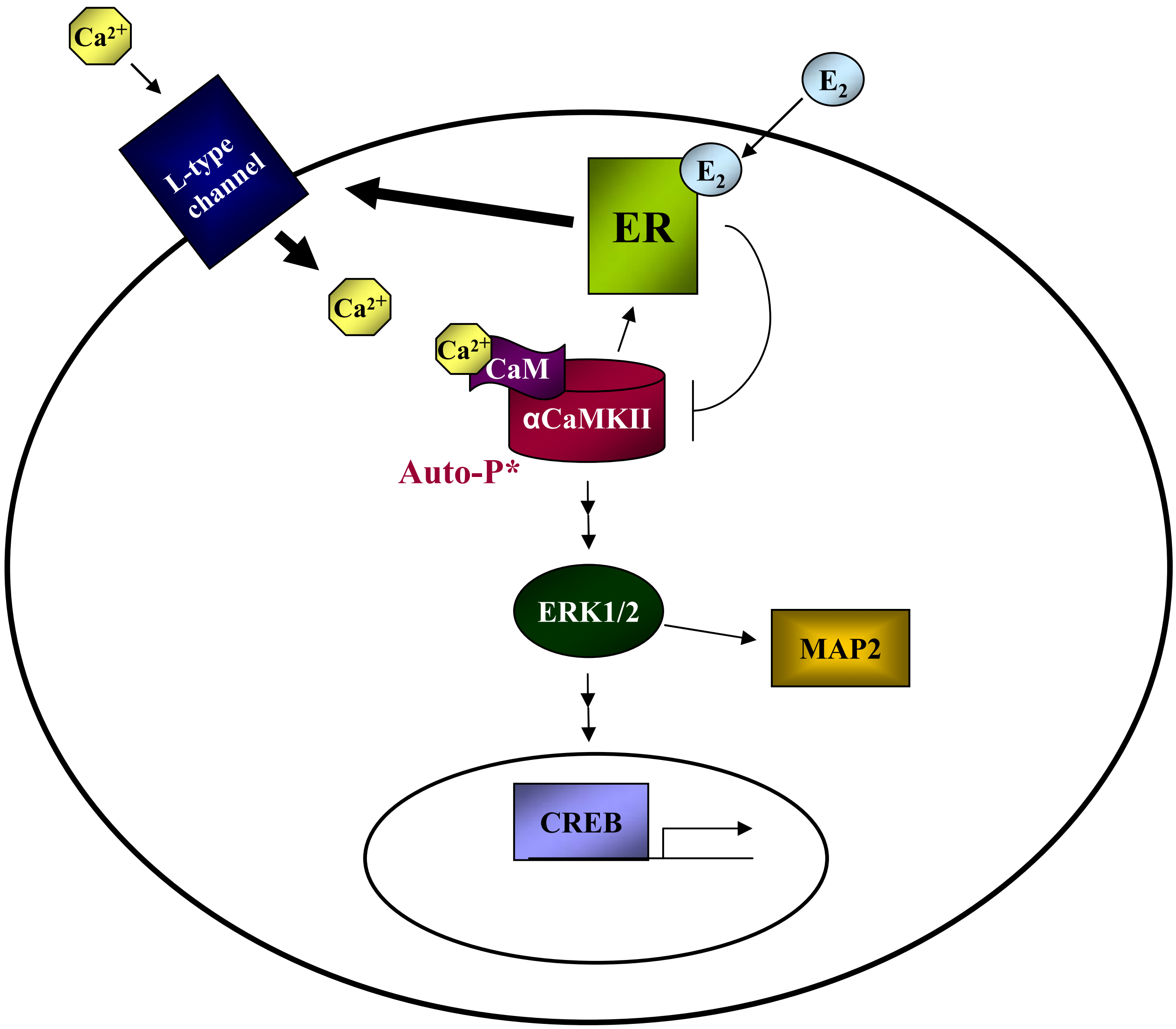
IF: MAP2

B. Neurite outgrowth of primary hippocampal neurons

	Mean Outgrowth Length (um)	Mean Primary Processes	Mean Branches	Straightness
Vehicle	73.91±21.71[a]	2.89±0.39[b]	2.40±0.84	0.84±0.006
E2	147.32±20.97	4.98±0.26	4.02±0.95	0.83±0.007
E2+KN62	65.05±19.90[a]	2.92±0.70[b]	1.24±0.60[c]	0.81±0.03

Figure 13. E2-induced αCaMKII activity influences neurite outgrowth of primary hippocampal neurons in a CaMKII-dependent manner. (A) Embryonic primary hippocampal neurons were treated 24 hours after plating with vehicle (0.1% DMSO) or KN-62 (10uM) and 2 hr later, vehicle (0.01%EtOH) or E2 (1nM) was added. Neurons were treated again 24 hr later and then fixed 24 hr post-2nd treatment and immunofluorescence for MAP2 was performed. 16 sites per well were imaged and analyzed for neurite outgrowth. **(B)** Mean outgrowth length, mean primary processes, mean branches, and outgrowth straightness were measured using the neurite outgrowth module included in the MetaXpress software package (Molecular Devices). Data are presented as the mean measurement ± S.D. *a)* mean outgrowth length, $p<0.05$; *b)* mean primary processes, $p<0.05$; *c)* mean branches, $p<0.04$, all relative to E2.

Figure 14



Ca2+
L-type channel
E2
E2
ER
Ca2+
Ca2+
CaM
αCaMKII
Auto-P*
ERK1/2
MAP2
CREB

Figure 14. Model of E2-induced αCaMKII signaling. E2 activates ERα, elevating intracellular Ca^{2+} levels by inducing Ca^{2+} influx via L-type Ca^{2+} channels. The Ca^{2+} spike is detected by αCaMKII, which indirectly phosphorylates ERK1/2 after being activated. Active ERK1/2 then results in the phosphorylation of CREB and MAP2. The ERα-αCaMKII interaction attenuates the ability of E2 to stimulate kinase autophosphorylation even though the receptor is initially required for E2 to induce αCaMKII activity. However, it appears as though the activating signal of E2 (thick arrows) supercedes the negative effect of ER since there is a clear, positive downstream response to E2-activated αCaMKII.